

DIETARY PROVOCATION OF REAGINIC  
ALLERGY IN YOUNG RATS

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Abstract

Atopic allergies are common and important causes of childhood morbidity. Treatment for conditions such as eczema, asthma and rhinitis is usually symptomatic and attention has therefore turned to their possible prevention. Though the genetic predisposition to atopic allergies is not in doubt, their provocation by environmental factors, as shown by a number of clinical studies, suggests that non-genetic factors may moderate the expression of atopic symptoms. One such factor is exclusive breast feeding, protecting the human infant against sensitization to several recognized antigens; an antigen non-specific effect. In contrast, other studies have shown that infants fed with cows milk preparations have more atopic disease and more immunoglobulin E antibody (IgE), though many do not produce an IgE response to cows milk proteins. Therefore it is not established from the clinical data whether cows milk feeds themselves provoke allergies, or if removing the protective effect of breast milk results in sensitization. Furthermore, feeding supplements of cows milk to a breast fed baby is a common practice, and its immunological effect is uncertain, though clinical evidence suggests that such a feeding regimen fails to protect against atopic allergies.

Jarrett and her colleagues have characterized IgE responses in Hooded Lister rats and this species was chosen for my investigations to determine if supplements of cows milk fed to suckling rat pups modifies the subsequent IgE antibody response to ovalbumin; this antigen non-specific effect was demonstrated. Supplements of a cows milk-based preparation increased the IgE and IgG response to injected ovalbumin. The effect was antigen dose dependent, more evident when a small dose was injected than with a higher

dose which produced comparably high IgE responses in both supplemented and unsupplemented animals. In contrast to the effect with the low dose, a higher dose reduced the IgG antiovalbumin response in supplemented rats; the explanation for this is unknown. One suggested mechanism of the antigen non-specific effect of cows milk feeds in human infants is that of disturbance in intestinal bacterial colonization, releasing endotoxin adjuvant into the circulation; my experiments in rats lend some support to this concept. The supplementary feeds with a cows milk preparation did not provoke an antibody response to cows milk proteins, suggesting that the detrimental effects of cows milk feeds are not confined to milk protein antigens; as in human infants, they potentiate sensitization to several recognized antigens. It is clear that such an effect is not just that of removing the protection against atopic allergy afforded by breast milk. Supplementary feeds to suckling rat pups did not influence the previously described antigen specific suppression of IgE response in the offspring of sensitized females; I have shown that suppression results from passive transfer of maternal antibody to the offspring. The relevance of this to human atopic allergy is uncertain and there is, as yet, no evidence that allergic mothers protect their babies against sensitization.

The IgE antibody response to immunization regimens previously described for eliciting such responses in adult outbred Hooded Lister rats were studied in the young rat by paper radioallergosorbent test and by passive cutaneous anaphylaxis. The factors which initiate the allergic response in the young rat may well also apply to the origin of atopic allergy in human infants. Allergic rats demonstrate anaphylaxis with intravenous antigen challenge and increased antigen absorption with oral antigen challenge; these effects parallel closely the clinical

effects of human reaginic allergy. The results of these studies support the view that dietary supplements fed to suckling mammals have important immunological consequences.



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## Chapter 1

### REVIEW OF THE LITERATURE

#### HUMAN ATOPIC ALLERGY

The recognition that allergic hypersensitivity was associated with human diseases such as eczema, asthma and rhinitis, led to the clinical description of atopic allergy (Coca, Cooke, 1923). Affected individuals have local immediate hypersensitivity reactions, shown by positive prick tests, and specific immunoglobulin E antibody against certain antigens (Wide, Bennich, Johansson, 1967). The antigens are ubiquitous yet only some people develop atopic allergy. It is suggested that atopic individuals are predisposed to sensitization as a consequence of genetic and environmental influences. Family studies have indicated a polygenic inheritance (Cooke, Wanderveer, 1916) (Turner, Rosman, O'Mahony, 1974) and this is supported by the association of atopic allergies with positive prick tests and with certain groups of histocompatibility antigens (Soothill, Stokes, Turner et al. 1976). The IgE response to ragweed pollen was shown to be closely linked with the HL-A7 haplotype (Marsh, Bias, Hsu, 1973), suggesting one instance of genetic control of allergy by immune response genes (Benaceraff, McDevitt, 1972), but this type of genetic regulation does not satisfactorily explain the multiple allergies of atopic patients. Atopic manifestations are related to certain immunodeficiencies (Soothill, Stokes, Turner, Norman, Taylor, 1976), for example, with transient immunoglobulin A (IgA) deficiency in the infants of atopic parents (Soothill, 1976), and patients with IgA deficiency develop antibodies to food antigens more frequently than the normal population (Buckley, Dees, 1969). When antigen is first

ingested it may enter the bloodstream (Walker, Isselbacher, Bloch, 1975) and so result in sensitization and immune exclusion (Swarbrick, Stokes, Soothill, 1979) (Rothberg, 1969). Antigen contact in an adverse form may instead provoke an IgE response particularly in the genetically predisposed infant (Walker, 1978: Editorial comments, Lancet, 1979). *In vitro* studies have shown that IgE responses are controlled by thymus-derived (T) lymphocytes (Ishizaka, Adachi, 1976) and there are clinical observations of T cell deficiency states associated with excessive IgE production (Kikkawa, Kamimura, Mamajima, 1973) (Church, Frenkels, Wright, Bellanti, 1976). In atopic individuals, *in vitro* studies have shown abnormalities in the population of T suppressor cells controlling for IgE synthesis (Buckley, Becker, 1978) (Holt, Turner, Holt, 1981). Such abnormalities may be inherited since they have been demonstrated in the offspring of atopic parents (Stannegård, Stannegård, 1979) (Juto, Stannegård, 1979) thereby predisposing the offspring to atopic allergies. However, the observation that histamine inhibits activation of T cells (Stannegård, Stannegård, 1977) raises the possibility that these abnormal T cell populations are secondary to the atopic disease. The subject has been reviewed in detail (Stannegård, Stannegård, 1979).

It is clear that the young infant is especially vulnerable to sensitization and subsequent clinical atopy (Soothill, Stokes, Turner, Norman, Taylor, 1976). Such sensitization may result from certain deficiencies of the neonatal immune system leading to exuberant IgE production (Kjellman N-IM, 1976). That adverse antigen exposure in the neonatal period is harmful is suggested by observations of neonatal pollen exposure and subsequent allergy to inhaled antigen (Bjorksten, Suoniemi, Koski, 1980). In addition, exposure to food antigen is also important in the provocation of eczema (Atherton, Sewell, Soothill, Wells,

Chilvers, 1978) and the immature gastrointestinal mucosa of the newborn readily permits antigen uptake (Eastham, Lichusco, Brody, Walker, 1978). Absorption of these antigens and their presentation to the immune system are of relevance to the pathogenesis of atopic allergy. It is likely from evidence in animals, that antigen exposure first occurs during intrauterine life (Brambell, Hemmings, 1949), and there is evidence that human neonates absorb intact protein from the gastrointestinal tract (Rothberg, 1969), yet such antigen contact does not usually sensitize the individual, possibly because of tolerance induction (Andre, Heremans, Vaerman, Cambiaso, 1975) (Lancet editorial, 1981). In genetically predisposed infants, sensitization readily occurs and they are not completely protected by breast-feeding, possibly because intact macro-molecules such as cows milk protein are secreted in human milk (Bjorksten, Sotarinen, 1978) (Jakobssen, Lindberg, 1977). The possibility that atopic individuals have an intrinsic mucosal defect (Schwartz, Leskowitz, Lowell, 1968) is not supported by histological evidence, nor by the greater prevalence of insect sting allergy (injected antigen) in the atopic population (Miyachi, Lessof, Kememy, Green, 1979).

The influence of environmental factors on the genetically predisposed preatopic infant is also suggested by the association of month of birth with the development of atopy (Soothill et al. 1976) (Morrison-Smith, Springett, 1979), and by the nonconcordance for atopic allergy in identical twins (Wuthrich, Baumann, Fries, Schnyder, 1981). Epidemiological studies have demonstrated that one such environmental influence, that of infant feeding, has important effects (Grulee, Sanford, 1936) (Glaser, Johnstone, 1953) (Johnstone, Dutton, 1966). Prospective studies have established that exclusive breast-feeding protects the genetically susceptible infant (Mathew et al. 1977) (Saarinen, Kajosaari, Bachman, Siimes, 1979).<sup>(a)</sup> The mechanism

of protection is uncertain but it is likely to be more complex than antigen avoidance in the first weeks of life because it prevents IgE responses to a wide range of environmental antigens (Chandra, 1979). The possibility must be considered that antigen avoidance by dietary elimination in early infancy merely postpones rather than prevents allergy (Saarinen, Kajossari, 1980). However, this prospective study of allergy to fish and citrus fruits does not permit firm conclusions to be drawn about the possible protective effects of breast feeding on subsequent development of food allergies in later infancy, because many of the studied infants had also received some feeds of cows milk.

Breast-fed infants have lower serum IgE immunoglobulin values than do bottle-fed ones, though the difference is not explained by IgE antibody to cows milk proteins (Saarinen, Bjorksten, Knekt, Siimes, 1979)<sup>(b)</sup>. Breast milk may protect through its demonstrable effects on immune responses, for example in the promotion of IgA synthesis in the neonate (Roberts, Freed, 1977) though this finding was not confirmed by a later study of infants in the post-neonatal period (Gross, Buckley, 1980). The effect of breast milk on neonatal lymphocyte responses has been shown *in vivo* (Schlesinger, Covelli, 1977) and *in vitro* studies (Pittard, Bill, 1979). In addition, thymus-derived suppressor lymphocytes, controlling IgE synthesis, were reduced in the breast-fed offspring of atopic fathers but not of atopic mothers (Juto, Stannegård, 1979). However, there are small amounts of cows milk protein in breast milk which can produce an IgE response (Bjorksten, Sotarinen, 1978). They may also cause gastrointestinal symptoms in breast-fed babies (Jakobssen, Lindberg, 1977) (Evans, Fergusson, Allardyce, Taylor, 1981) but these symptoms may not be the result of sensitization to cows milk protein. Exclusive breast feeding is not entirely protective, but infants fed with mixtures of breast and cows milk develop more atopic symptoms than those entirely breast fed (Hide, Guyer, 1981). The possibility that breast milk supplemented by small amounts

of cows milk is sensitizing has been confirmed (Firer, Hosking, Hill, 1981), but this study was confined to cows milk allergy and did not explore the effect of antigen dose (cows milk) on sensitization to unrelated antigens. The relevance of cows milk allergy to other infant atopic allergies is uncertain because only a minority of cows milk allergic infants have positive prick tests and symptoms resolve in most before two years of age (Jakobssen, Lindberg, 1979). Cows milk feeds may be intrinsically allergenic even when ingested with breast milk. Bottle-fed or supplementary-fed infants have more atopic disease (Chandra, 1979) and more IgE antibody than do breast-fed ones (Saarinen et al. 1979).<sup>(b)</sup> The sensitization resulting from cows milk feeds is antigen nonspecific because many such infants do not produce IgE antibody to cows milk proteins (Kletter, Gery, Freier, Noah, Davies, 1971) (Saarinen et al. 1979).<sup>(b)</sup> The mechanism of sensitization is uncertain but cows milk feeds predispose to gastroenteritis (Gordon, Chitkara, Wyan, 1963) resulting in mucosal damage and greater antigen absorption (Grusky, Cooke, 1955) (Iyngkaran, Dans, Robinson, Boey, Sumithran, Yadav, Cam, Puthuchear, 1979). In addition, absence of protective humoral factors in the diet of non-breast-fed infants facilitates the production of enterotoxin in the intestine (Bullen, Tearle, Steward, 1977) (Smith, C.W., 1973) (Stoliar, Kaniecki-Green, Pelley, Klaus, Carpenter, 1976). The adjuvant properties of endotoxin *in vitro* are well known (Morrison, Ryan, 1979). It is suggested that absorption of endotoxin together with dietary antigen provokes an IgE response (Editorial comment, Lancet, 1979). The interaction between genes and the environment in the manifestation of atopic allergy is apparent but the relative effects and mechanisms of action are not fully resolved by clinical studies.



## IgE RESPONSES IN EXPERIMENTAL ANIMALS

Rat pups, like human newborn, are entirely deficient in IgE immunoglobulin which does not cross the placenta. (Ammann, Stehm, 1966) (Karlsson, 1979). They absorb immunoglobulin of the IgG class across the gut mucosa in the first twenty days of life (Good, Papermaster, 1964) and also acquire immunity by the active synthesis of immunoglobulin, including IgE, which has been detected from the age of fifteen days (Karlsson, 1978), by sensitive radioimmunoassay methods for IgE and IgG antibody (Karlsson, Ellerson, Haig, Jarrett, Bennich, 1979)<sup>(a)</sup> (Bennich, Ellerson, Karlsson, 1978). The IgE response to injected antigen is of small magnitude and short duration (Binaghi, Benacerraf, 1964). However, Jarrett (1974) showed that mature rats of the Hooded Lister strain readily produced an IgE and IgG antibody response when small doses of antigen were injected intraperitoneally with heat killed Bordetella Pertussis. The IgE response was increased by a second injection of antigen without adjuvant. The size of the secondary response was determined by the amount of antigen given in the first immunisation: for ovalbumin the dose range was from 0.1 µg to 100 µg. The size of the secondary response was similar with antigen doses between 0.001 µg to 100 µg, but larger doses produced fatal anaphylaxis. Further IgE responses could not be detected in the sensitized rats by subsequent injections of antigen. Larger doses of antigen (>100 µgs) injected in the primary immunisation also suppressed the IgE response and this may have resulted from the induction of T suppressor lymphocytes (Jarrett, 1977). There is some evidence that the dose of antigen influences the IgE response in human infants, at least with respect to allergy to cows milk proteins (Firer, Hosking, Hill, 1981) therefore some factors controlling IgE production may be similar in man and rat.

Most rat strains other than the Hooded Lister less readily produce IgE and so are less suitable to demonstrate the effects of experimental manipulations on IgE response. Administration of antigen in small doses by the oral route also provokes an IgE response in the rats similar to that of injected antigen, though adjuvant is necessary; the adjuvant may be administered orally (Bazin, Platteau, 1976) or intraperitoneally (Jarrett, Haig, McDougall, McNulty, 1976) concurrently with the oral antigen. Jarrett used a range of ovalbumin dose similar to that given by the intraperitoneal route (1  $\mu$ g - 100  $\mu$ g) and the kinetics of the IgE antibody response were the same whether the antigen was injected or ingested. Bazin and Platteau (1976) used a different rat strain (Lou/M/WSL) and found that primary immunisation with ovalbumin doses of 10 to 100 mgs. were most effective. Rat IgE immunoglobulin levels increase in serum from birth to six weeks of age when adult values are attained (Karlsson, 1978). The IgE antibody response to body weight-related doses of antigen with adjuvant were greatest between one or two months of age, and thereafter decreased progressively (Pauwells, Bazin, Platteau, Van der Straeten, 1979). This interesting observation suggests that the young rat is especially predisposed to develop reaginic antibody with appropriate immunisation.

In rats, there is evidence that IgG antibody inhibits the production of reaginic response (Tada, Okumura, 1970). However, Jarrett and others (1974) have argued that this is not the principal mechanism controlling IgE antibody responses since antibody of both immunoglobulin classes is simultaneously produced by appropriate immunisation, and suggest that cellular regulation of antibody response predominates.

The cellular mechanisms controlling IgE responses have been explored in experiments with inbred murine strains. Manipulation of lymphocyte responses by irradiation, immunosuppressive drugs, thymectomy or antilymphocyte serum increased the magnitude of IgE response (Tada, 1975). Total IgE and IgE antibody responses in rats were increased by adoptive transfer of thoracic duct lymphocytes from immunized animals of the same inbred strain. Fractionation and transfer of TDL's without surface antigen (presumably Thymus-derived) increased total antigen non-specific IgE immunoglobulin (Narva, Miller, Hall, Jarrett, 1981). The antigen specific response was only increased by the additional transfer of primed B cells.

Murine IgE responses were suppressed or enhanced by humoral factors passively transferable in serum or ascitic fluid (Katz, 1979). These factors are not immunoglobulin and modify antibody responses in an antigen non-specific way. The IgE responses in mice differ from human allergy in at least two important respects. Sensitization requires injected adjuvant and does not occur spontaneously as in man, and classes of immunoglobulin other than IgE are not increased whereas sensitization in allergic humans produces antibody of all classes of immunoglobulin (Platts-Mills, Snajdr, Ishizaka, Frankland, 1978). The suckling young of some species, such as guinea pigs (Anderson, McLaughlan, Devey, Coombs, 1979) and calves (Kilshaw, Slade, 1980), have been sensitized with ingested foreign protein without adjuvant. In rats, the effect of antigen contact before weaning has not been studied before now, but clinical evidence suggests that the ingestion of dietary antigen such as cows milk during the suckling period influences subsequent IgE responses (Juto et al. 1980) (Saarinen et al. 1979)<sup>(b)</sup>.

Other mammalian species demonstrate different patterns of absorption of macromolecular proteins in the neonatal period; the absorption of gammaglobulin has been most commonly studied. The calf and piglet

acquire no gammaglobulin during intrauterine life but receive large amounts during suckling which is absorbed intact together with other macromolecular proteins (Brambell, F.W., 1966)(Lecce, 1966).

Functional closure of the gut at weaning results in intestinal impermeability to these proteins. Rodents have an extended period of selective absorption of gammaglobulin from maternal milk (Morris & Morris, 1976)(Walker, Isselbacher, 1974). There are receptors for gammaglobulin on the surface of gut mucosal cells (Borthwhistle, Kubo, Brown, 1977). During pinocytotic ingestion of macromolecular proteins by the intestinal epithelium of suckling rats, the absorbed IgG attached to the specific receptor is protected from lysosomal digestion and is passed by exocytosis into the circulation. Absorption of other intact proteins almost certainly occurs; as much as 2% of labelled bovine serum albumin fed to adult rats can be detected in portal and peripheral blood (Warshaw, Walker, Isselbacher, 1974). There is evidence that the intestinal transport mechanism for gammaglobulin non-specifically transports other proteins (Smith, Burton, Munn, 1979). Oral or systemic immunization reduces the uptake of proteins (Swarbrick, Stokes, Soothill, 1979)(Walker, Wu and Isselbacher, 1975b)(Hemmings, Wood, 1975), but systemic anaphylaxis increases intestinal protein absorption (Bloch, Bloch, Stearns, Walker, 1979). However, these observations may well not be relevant to the pathogenesis of human atopic allergies since antigen handling by the human neonatal gut has not been studied extensively and there is no known transport mechanism for human milk immunoglobulin.

Rat pups have been successfully reared with a cows milk-based feed (Hall, 1975) and such feeds may have effects on IgE responses when compared with normally suckled animals because the chemical and immuno-

globulin content of the rat milk differs from that of cows milk derived diets (Luckey, 1954)(McGhee, Michalek, Ghanta, 1975). Such diets change intestinal microflora in human infants though there is no data in other animals. Rat colostrum promotes growth of the intestine (Heird, Hansen, 1977)(Younoszai, Ranshaw, 1973) and provides immunoglobulin for the suckled pup (Mackenzie, 1972). In addition, rat milk may promote the normal 'gut closure' to immunoglobulin and other macromolecules at twenty days of age (Rodewald, 1973). It is possible that supplementary feeds of cows milk might disturb some of these functions of the rat milk, and this might influence IgE responses in the rat pups. The diet of suckled rats fed supplements of cows milk preserves some of the beneficial effects of rat milk such as milk immunoglobulin, but human infants, predisposed to atopy, develop allergic symptoms following a diet of breast and cows milk (Chandra, 1979)(Firer, Hosking, Hill, 1981). Therefore, the effects of supplementary feeds fed to rat pups was investigated.

Clinical observations in human infants that exclusive breast feeding protect against eczema suggests that the effects of the cows milk-based diet are likely to be antigen non-specific, since other food antigens, such as egg are also important in the provocation of eczema (Atherton, Sewell, Soothill, Wells, Chilvers, 1978). In rats, Jarrett and Hall (1979) showed that preimmunization of mated females with ovalbumin prevents an IgE anti-ovalbumin response in their suckled offspring, and cross fostering experiments demonstrated that this was a function of milk. This effect was antigen specific; IgE responses to other antigens were not affected, unlike the impression of the effect of breast milk in the human infant. The effect in rats may be due to absorbed IgG antibody, and human infants do not absorb maternal IgG from the milk. There is no evidence that antigen-specific suppression of IgE responses occurs in the breast-fed infants of atopic mothers.

METHODS OF FEEDING MILK TO RAT PUPS

The physical and chemical characteristics of rat milk were defined by Luckey et al. (1954). Early attempts to feed rat pups with a non-rat milk were unsuccessful. Abdominal distension, diarrhoea, tremulousness and failure to thrive were observed in the recipients (Dymsza, Czaki, Miller, 1964). Rat pups will not suckle from a non-maternal milk source and they require gavage feeding with a fine orogastric tube. Milk preparations modified to resemble rat milk by correction of osmolarity, vitamin and aminoacid content were more successful in promoting growth in rat pups (Miller, Czaki, 1967) (Messer, Thomas, Terrasa, Dallman, 1969). Failure to thrive in rat pups frequently precludes the use of a diet derived completely from cows milk; there is only one report of successful weaning and rearing (Hall, 1975) and in this experiment milk was fed by continuous infusion via gastrostomy tube. This feeding method was considered insufficiently physiological to allow satisfactory comparison with suckled control animals. Inadequate milk intake partly accounts for poor weight gain, but there are also non-caloric factors in rat colostrum which promote growth of the gastrointestinal tract and thereby increase food absorption in suckled rats (Younoszai, Ranshaw, 1973). This property of colostrum has also been observed in other suckling mammals (Widdowson, Colombo, Artavanis, 1976).

The gut mucosa of rat pups has immunoglobulin Fc receptors (Borthistle, Kubo, Brown, 1977), and these bind immunoglobulin complexed with food protein, facilitating food absorption by pinocytosis (Abrahamson, Powers, Rodewald, 1979). These observations suggest that some colostrum is essential for normal growth and immunity. The feeding experiments planned in my studies were based on supplements of cows milk based preparations derived from previous reports.



The food requirements of suckling rat pups have been determined from fluid balance studies and serial measurements of weight gain (Morag, 1970). Suckling rats ingest approximate volumes of 0.5 ml of milk/g body weight/24 hours (Reddy, Donker, Linnerud, 1964). Gastric volume determined by gavage ranged from 0.25 mls to 3.5 mls between birth and weaning (twenty-one days of life). Feeding intervals were calculated from these requirements. The regimen so derived paralleled closely observation of normal suckling behaviour.

#### ADJUVANTS IN IgE RESPONSES IN ANIMALS

Parasitic infestation in man and other species provokes an intense IgE antibody response; not all this antibody is directed against the parasite so there is evidence of non-specific stimulation of IgE synthesis (Orr, Blair, 1969). This adjuvant property of the parasite antigen has been shown in experiments in rats to produce high titres of IgE antibody to other antigen. For example, the IgE antiovalbumin response was increased by administration of ovalbumin together with *Nippostrongylus Brasiliensis* (Jarrett, Stewart, 1972). Another adjuvant, *Bordetella Pertussis* apparently works rather differently (Jarrett, Hall, Karlsson, Bennich, 1980) (Ho, Morse, Kong, 1981). Injection of Hooded Lister rats with heat-killed *B. Pertussis* with small doses of antigen (1.0 ng-1.0 µg ovalbumin) produced IgE and IgG antiovalbumin antibody without an associated increase in non-specific serum IgE immunoglobulin, and antibody titres to other sensitizing antigens were not affected (Jarrett, Stewart, 1974). IgE antibody responses were not detected when the same dose of antigen was administered without the pertussis. A specific adjuvantizing protein, Pertussigen, has been identified in extracts of heat-killed pertussis (Sadowski, Robbins, Munoz, 1979). Pertussis is an effective adjuvant when given with a primary or secondary immunization (Reed, Benner, Lockey,

Enta, Makino, Carr, 1972). The effect of pertussis adjuvant when given with both the first and second immunization has not been reported in rats, though reproducible adjuvant effects on IgE responses have been observed in mice after repeated administration of antigen in small doses with Alum adjuvant (Levine, Vaz, 1970). Unlike rats, atopic humans spontaneously produce IgE antibody to certain antigens and the antibody response is increased with continued exposure to antigen (Viander, Koivikko, 1978). It has been suggested that naturally occurring adjuvant such as E Coli endotoxin, absorbed from the gut, may increase reaginic responses in susceptible groups, i.e. babies fed cows milk preparations (Lancet Editorial, 1979). The adjuvant properties of bacterial endotoxin in murine and rodent species have been reviewed by Morrison and Ryan (1979). The principal components of the endotoxin, Lipid A and lipopolysaccharide (L.P.S) influence a broad spectrum of cellular and humoral immune responses. B lymphocytes have receptors for the lipid A moiety (Forni, Coutinho, 1978) and endotoxin abrogates B cell-mediated tolerance in rats (Ornellas, Sanfillippo, Scott, 1970). LPS facilitates the helper rôle of T lymphocytes in response to antigen challenge (Hamoaka, Katz, 1973) (Morrison, Ulevitch, 1978).

It is evident from experiments in guinea pigs that endotoxin potentiates specific and non-specific immune responses including those of IgE antibody (Perini, Mota, 1973). The hypothesis that cows milk feeds increase IgE responses in human infants may also apply to rat pups fed supplements of cows milk. The anamnestic response of rats to *N. Brasil-*ensis does not protect against IgE antibody response to subsequent immunization with conventional antigen such as ovalbumin (Jarrett, Mackenzie, Bennich, 1980).



Fatty acid esters, particularly those with a large unsaturated fatty acid component, appear to have adjuvant properties (Bomford, 1981). Cows milk-based diets have a different fat composition to that of breast milk but it is not known if this difference has relevance to the postulated adjuvant properties of cows milk-based diets.

I have investigated the adjuvant effects of Bordatella Pertussis and bacterial lipopolysaccharide on the IgE responses to injected and to ingested ovalbumin in young Hooded Lister rats. The postulated effects of dietary modification by supplementary feeds of a cows milk preparation on these responses was explored.

SUMMARY OF CHAPTER 1

Clinical observations of atopic disease emphasize the special vulnerability of the human infant to the development of IgE mediated hypersensitivity. There is substantial evidence that the environment influences the development of atopic disease in genetically predisposed infants. Many potentially protective substances are found in breast milk which modify intestinal microflora, and restrict the production of enterotoxin. In addition, breast milk is a good antigen-avoidance diet. Replacement of this diet with cows milk preparations increases the prevalence of atopic allergy. There are several possible mechanisms for this, such as excessive antigen exposure, absorption of endotoxin adjuvant from intestinal enterobacteria and loss of the immune properties of breast milk. The evidence from clinical observations is inconclusive; breast milk is not completely protective and cows milk feeds are not always harmful. The mechanism by which cows milk feeds appear to provoke atopic allergy is not likely to be antigen-specific since affected infants do not usually produce reaginic antibody to cows milk proteins.

Studies of IgE antibody responses in rodents have contributed usefully to our knowledge of the control of IgE synthesis and the provocation of human allergy. The following investigations were performed with an outbred rat strain that readily produces IgE following appropriate immunization. The experiments were designed to parallel clinical situations and the results will be discussed in relation to their possible clinical significance.

## Chapter 2

### PREPARATIVE AND ANALYTICAL METHODS

#### INTRODUCTION

Antibody responses were produced in rats by several methods. For studies of IgE responses, animals were injected or fed with ovalbumin together with Bordetella pertussis adjuvant: the methods described are identical to those of Jarrett et al. (1974, 1976). Animals so immunized were used in subsequent experiments to explore the effects of feeding with cows milk-based supplements. Others were immunized with ovalbumin in Freund's complete adjuvant (FCA): this produced haemagglutinating antiovalbumin antibody in high titres but IgE responses were not detected. Some of these animals and some with IgE antiovalbumin antibody were challenged by intravenous injection with ovalbumin, to contrast anaphylaxis symptoms in the two groups. Pooled serum from rats immunized with antigen in FCA was purified by ion exchange chromatography to obtain an IgG fraction containing antiovalbumin antibody. This preparation was fed to young rats to determine its effect on subsequent IgE responses to ovalbumin.

IgE antibody and total IgE to ovalbumin and to  $\beta$  lactoglobulin were determined by radioabsorbent test (RAST) and radioimmunosorbent test (RIST) using antigen-coated paper discs (Karlsson et al. 1979<sup>(a)</sup>). The RAST was validated by correlation of values of positive serum with titres obtained by passive cutaneous anaphylaxis. Results were expressed as a percentage of the binding relative to counts bound by newborn rat serum, which contains no IgE (Karlsson, 1978). Batch variations of the method was observed and described.

Haemagglutinating antibody was detected by antigen-coated erythrocytes after absorption of test serum with uncoated red cells (Koffler, Wick, 1977). The method was modified to estimate IgG antibody by pre-treatment of serum with DL Dithiothreitol (Olsen et al, 1976). IgE and IgG antibody were the principal responses to immunization with antigen and pertussis adjuvant found by Jarrett et al. (1974) in experiments with adult HL rats.

There are only a few reports of antibody responses to such immunization regimens in young Hooded rats (Pauwells et al. 1979)(Jarrett, Hall, 1980)(Karlsson, 1978) these were explored in my experiments because the effects of antigen contact early in life may parallel more closely the development of atopic allergies in human infants. Since feeds of cows milk-based preparations to young rats might produce an antibody response to cows milk proteins, the IgE and IgG anti  $\beta$  lactoglobulin response was studied. Such responses might be of relevance to cows milk protein allergy in human infants.

## IMMUNIZATION PROCEDURES

### Preparation of Antigen Solutions

5 x crystallised ovalbumin and  $\beta$ lactoglobulin were purchased from Sigma Chemicals. Stock solutions of antigens in concentrations of 10 mg/ml were prepared in 0.15N NaCL and stored at  $-70^{\circ}\text{C}$ . Dilutions at concentrations of 1 mg/ml, 100  $\mu\text{g/ml}$  and 10  $\mu\text{g/ml}$  were made in 0.15N NaCL immediately before administration to the rats.

### Adjuvants

An aqueous suspension of heat-killed Bordatella Pertussis ( $80 \times 10^9$  organisms/ml) was kindly provided by Wellcome Research Laboratories.

Bacterial lipopolysaccharide (Eschershia Coli. Sigma Chemicals) was freshly prepared before administration in concentrations of 1 mg/ml and 100  $\mu\text{g/ml}$  in 0.15N NaCL.

Freunds complete adjuvant (Wellcome) was emulsified with an equal volume of the antigen solutions before administration.

### Administration of Antigen and Adjuvant

Immunization by Intraperitoneal Injection: Volumes of antigen (0.1 ml), the pertussis adjuvant (0.12 ml) or the E.Coli lipopolysaccharide (0.1 ml) were drawn up in separate 1 ml disposable syringes. Antigen and adjuvant were separately injected via sterile 25FG needles into the peritoneal cavity of Hooded Lister rats (HL). Injection sites were chosen in the mid-line anterior abdominal wall and between umbilicus and pubis.

Oral Immunization: Weaning HL rats were fasted for four hours and immunized with 0.1 ml volumes of antigen introduced by an orogastric tube 3 cms. in length. Mature fasting rats were given antigen through a blunt metal cannula, 10 cms. in length, introduced into the stomach. Adjuvant was given either by the same route or separately by intraperitoneal injection.

Subcutaneous Immunization: Mature HL rats were immunized to produce an IgG antibody response with ovalbumin or  $\beta$  lactoglobulin emulsified in Freund's complete adjuvant, injected subcutaneously into the posterior cervical tissues, three times, at intervals of fourteen days.

Blood Sampling

Cardiac puncture: The method was used for rats aged six weeks or more. Animals were lightly anaesthetized by ether inhalation. A sterile 23FG needle was inserted under the xiphisternum and advanced dorsally into the heart when a free flow of blood was obtained. If the animals were not to be killed, a maximum volume of 2 mls. was removed. Samples were centrifuged, serum separated and stored at  $-70^{\circ}\text{C}$ .

Tailvein: Small volumes (maximum of 0.5 ml) of blood were taken from young rats by amputation of the terminal 1 mm of the tail, and 'milking' blood into a test tube.

Newborn HL rat serum: Newborn rats were lightly anaesthetized, decapitated and blood was pooled and centrifuged. Serum was stored at  $-70^{\circ}\text{C}$ . This serum does not contain rat IgE (Karlsson, 1978).

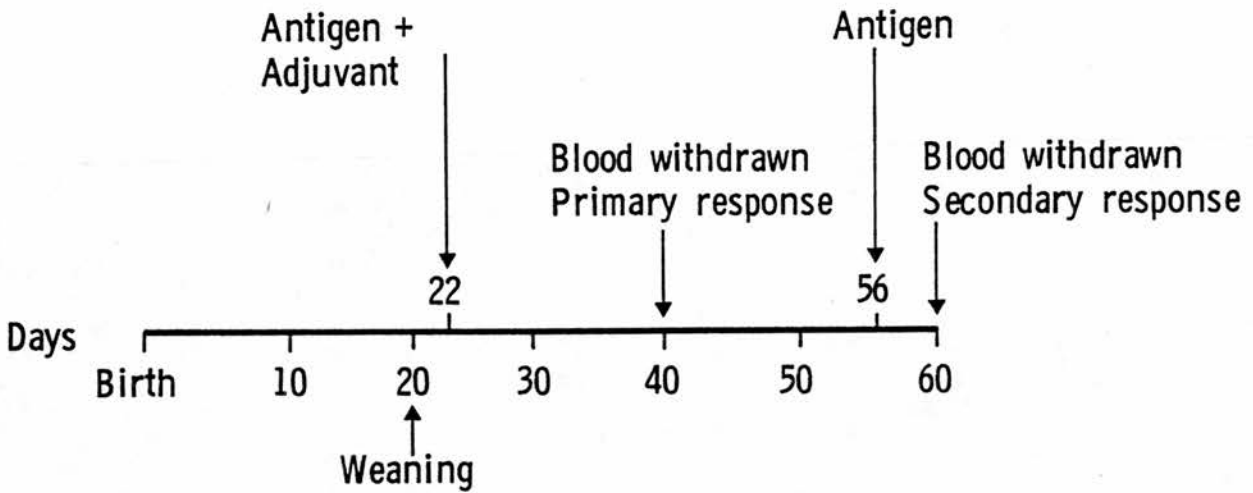
Immunization Schedule

Young HL rats were immunized as shown in the scheme (Figure 1). The time sequence follows that of Jarrett and Stewart (1974).

In some experiments blood was sampled at forty days to measure the primary IgE antibody response. Jarrett and Stewart (1974) have shown in mature rats of the same HL strain, that the secondary response is of greater magnitude and shorter duration than the primary one, the peak IgE antibody response is four days after the secondary immunization. Bordetella pertussis adjuvant was given with the first but not the second dose of antigen.

FIGURE 1:

IMMUNIZATION SCHEME IN YOUNG HL RATS FOR THE PRODUCTION OF IgE ANTIBODY, USING ANTIGEN AND HEAT KILLED BORDATELLA PERTUSSIS



IN VIVO MEASUREMENT OF IgE ANTIOVALBUMIN ANTIBODY

Passive cutaneous anaphylaxis was used to detect reaginic antibody in immune serum (Ovary, 1958). Unimmunized low IgE responding rat strains were used in the PCA assay to prevent blocking of cutaneous mast cell binding sites by intrinsic IgE antibody. Karlsson (1978) found no difference in PCA titres with recipient rats of low or high IgE responsiveness, so this consideration probably has no practical importance. Doubling dilutions of serum from sixty-day old rats immunized with ovalbumin and pertussis adjuvant in normal saline were prepared (Fig. 1).

0.1 ml. of each dilution was injected intradermally into the shaved dorsal skin of unimmunized adult PVG or Sprague Dawley (SD) rats (OLAC - Oxford Laboratory Animals Centre). The injection sites were equidistantly spaced 2 cms. from each other. The PVG or SD recipients were simultaneously injected intradermally with a control of 0.1 ml. pooled serum obtained from 18 unimmunized sixty-day old HL rats. They were lightly anaesthetized with ether after forty-eight hours and 0.25 ml of 1% ovalbumin together with 0.5 ml of 4% Evans Blue in 0.15N NaCl was injected via the tail vein. A circular zone of dense blue cutaneous staining developed at the site of positive reactions (Figure 2); the diameters were measured fifteen minutes after injection by superimposing a transparent plastic plate with circles of known diameter. Recipient animals were then killed and skin reactions confirmed by examination of the subcutaneous region (Figure 3). The reciprocal of the greatest serum dilution giving a positive reaction (stained zone  $> 5$  mm) was recorded as the PCA titre ( $-\log_2$ ). No reaction occurred at any of the control injection sites.

LEGEND TO FIGURE 2 AND FIGURE 3

Positive PCA reactions in 3 of the 4 injections of immune serum in doubling dilutions. Injection site of control serum not shown.

Subcutaneous region of the skin shown in Figure 2.



FIGURE 2:

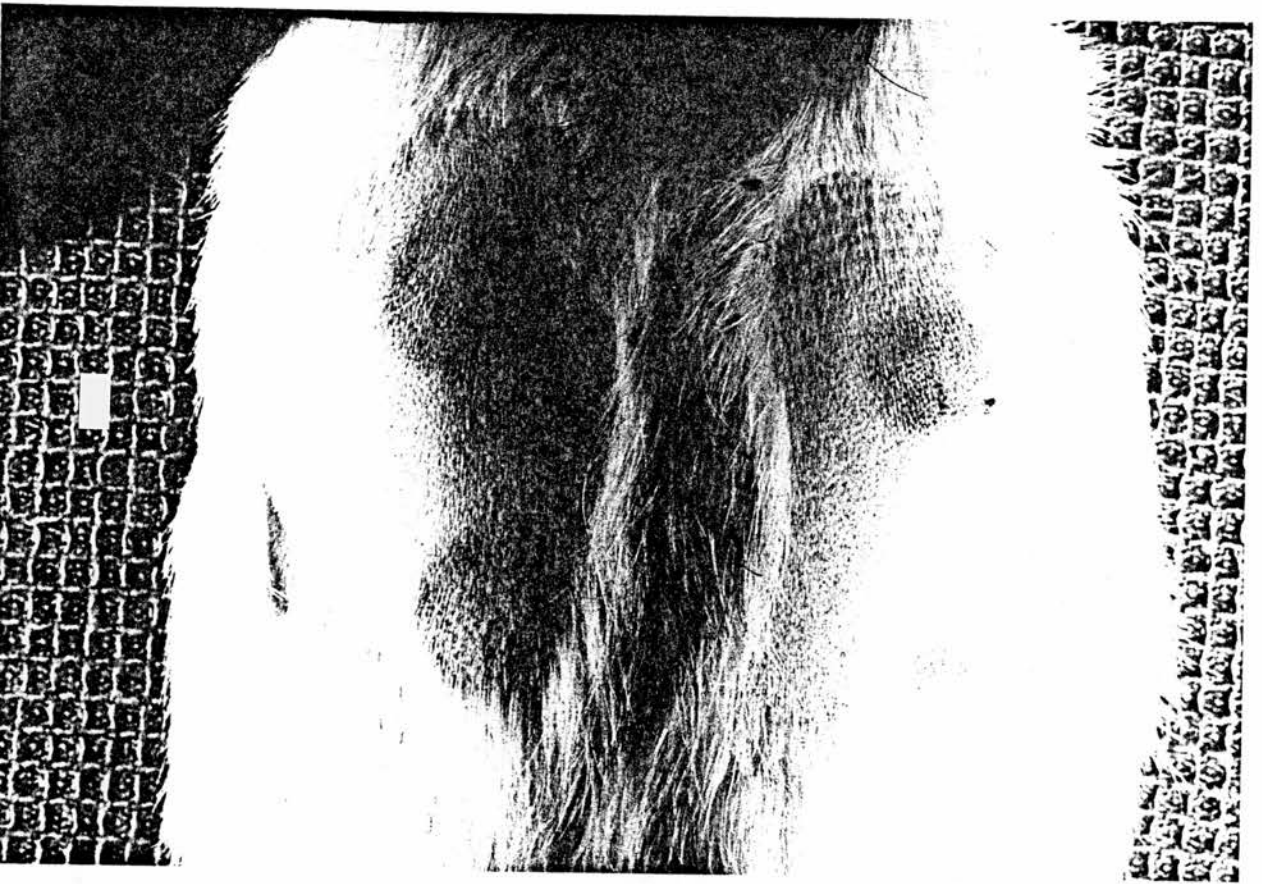


FIGURE 3:



IN VITRO ASSAY OF IgE ANTIOVALBUMIN ANTIBODY

Serum IgE antiovalbumin was determined by a paper radio allersorbent method (Karlsson, 1978)(Karlsson, Ellerson, Haig, Jarrett, Bennich, 1979)<sup>(a)</sup>(Figure 4). I used a rabbit anti-rat Fc epsilon antibody kindly donated by Dr. Jarrett. This reagent was prepared by Dr. Karlsson from rat IgE myeloma serum (Karlsson, 1978) and protein concentration after dialysis determined from absorbence at 280 mμ in a spectrophotometer. The optimum conditions for the test were determined, and the correlations with PCA titres were studied. Human sera containing IgE antiovalbumin antibody gave negative results in the assay.

Cyanogen Bromide Activation of Paper Discs

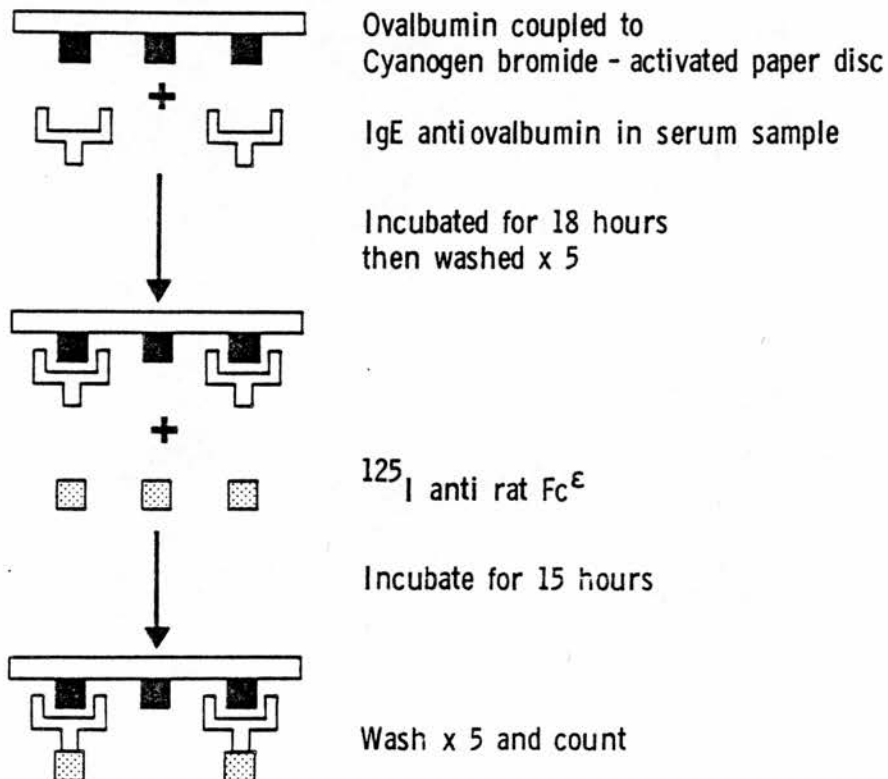
3G of discs were cut from filter paper (Whatman, 541) with a paper punch and washed twice in deionised water. 100 mls of a 5% solution of cyanogen bromide in water was dissolved by stirring in a fume cupboard for thirty minutes. This solution was added to the discs and maintained at a pH 10.5-11.0 by dropwise addition of 1M NaOH for twenty minutes. CnBr solution was decanted and the discs washed repeatedly in 5 litres of ice-cold 0.005 M NaHCO<sub>3</sub> pH 9.0. The activated discs were then immediately coupled to antigen, or washed in deionised water, dried in a dessicator and stored at +4°C.

LEGEND TO FIGURE 4

DIAGRAM OF THE PAPER RADIO ALLERGOSORBENT TEST (PRAST)

(From Karlsson, 1978.)

DIAGRAMMATIC SCHEME OF THE P.R.A.S.T.



### Coupling Antigens to Activated Discs

Carbonate/Bicarbonate coupling buffer was prepared (see Appendix, Reagents).

200 discs were added to 20 mls. of 1% antigen solution (Ovalbumin and  $\beta$  lactoglobulin) in coupling buffer in a universal container, and mixed on a rotating tray for eighteen hours at 4°C.

Discs were washed x 3 in 0.5M NaHCO<sub>3</sub> pH 9.0 to remove excess protein, and then mixed with 0.1M ethanolamine in coupling buffer for eighteen hours at 4°C.

Two further washes in 0.5M NaHCO<sub>3</sub> and three washes in 0.1M acetate buffer were performed. Finally, the discs were washed x 2 in incubation buffer (Appx.) dried and stored at 4°C.

Measurement of IgE immunoglobulin by radioimmunosorbent assay required discs coupled with purified rabbit anti rat IgE antibody. 100  $\mu$ g (250  $\mu$ l) of the antibody, kindly provided by Dr. Jarrett, were incubated with 200 activated discs in 20 mls of coupling buffer, then treated identically to the ovalbumin coated discs.

### <sup>125</sup>I Labelling of Anti Rat Fc<sup>E</sup> Antibody

Anti rat Fc<sup>E</sup> antibody solution containing 8.06  $\mu$ g protein/ $\mu$ l was divided into aliquots of 40  $\mu$ g (5  $\mu$ l). 25  $\mu$ l of 0.5M phosphate buffer pH 7.5 and 1 MC<sup>125</sup> was added to a glass tube placed in a bucket of ice. 40  $\mu$ g of anti rat Fc<sup>E</sup> was added to the mixture, rotated, and 25  $\mu$ l of Chloramine T 1 mg/ml in 0.5M phosphate buffer was immediately added.

After rotation for sixty seconds, 25  $\mu$ l of Sodium Metabisulphite 2 mg/ml in 0.5M phosphate buffer was added, followed by 10  $\mu$ l of 1% potassium iodide.

A sephadex 25G column (PD 10) was equilibrated with 20 mls 0.05M phosphate buffer and 0.3% BSA.

The iodinated protein was placed on the column and eluted with phosphate buffer 0.05M. Aliquots of six drops were collected. 100  $\mu$ l of a 1/100 dilution of each aliquot were counted in a Wallac gamma counter. Fractions containing the protein peak were pooled and used in the PRAST assay. TCA precipitation, determined before use, ranged from 80-95%.

#### PRAST Procedure

A paper disc and 100  $\mu$ l of incubation buffer were added to each of a rack of LP3 plastic tubes. 50  $\mu$ l of test serum or known positive serum (kindly supplied by Dr. Jarrett) or serum from newborn HL rat pups, were added to duplicate tubes. Two tubes with no added serum were also included.

After incubation on a rotater for eighteen hours at room temperature, discs were washed x 5 in incubation buffer, and 100  $\mu$ l of labelled anti  $\text{Fc}^{\text{E}}$  antibody diluted in diluent buffer, (see Appex.) were added, having a count rate of 15-25,000 counts/min/tube. 100  $\mu$ ls was set aside for total added counts.

Discs were incubated for a further eighteen hours on a rotator at room temperature, washed x5 in 0.15N Saline and counted. Results were expressed as counts bound by sample relative to counts bound by neonatal rat serum.

#### Sensitivity of the PRAST

Doubling dilutions of a positive reference serum (kindly supplied by Dr. Jarrett) having a PCA antiovalbumin titre of 10 ( $-\log_2$ ) and total IgE immunoglobulin concentration of 120 mg/litre were prepared in 10% unimmunized rat serum. IgE antiovalbumin antibody was assayed in duplicate by PRAST

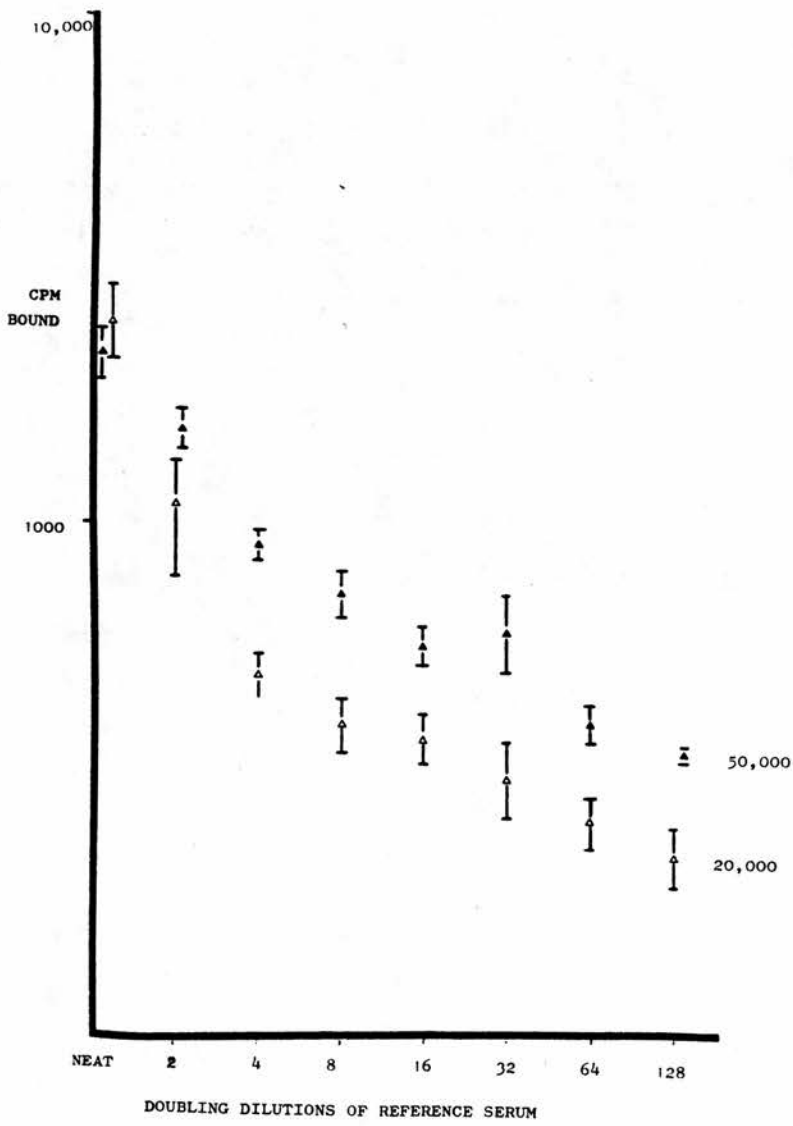
The effect of different concentrations of second antibody was determined with two dilutions of  $^{125}\text{I}$  anti rat  $\text{Fc}^{\text{E}}$  containing 50,000 cpm.  $100\ \mu\text{l}^{-1}$  and 20,000 cpm.  $100\ \mu\text{l}^{-1}$ . Mean and range of counts bound in the standard curve are shown in Figure 5. The assay was at least as sensitive as passive cutaneous anaphylaxis in the detection of extremely small concentrations of IgE antibody in the dilute reference serum. Assay sensitivity was not significantly improved by the higher concentration of labelled anti rat  $\text{Fc}^{\text{E}}$  antibody.

#### PRAST: Interassay Variation

Nine serum samples of known PCA titre and different IgE antiovalbumin concentration were compared in separate assays using different batches of ovalbumin-coupled paper discs.  $100\ \mu\text{l}$  of  $^{125}\text{I}$  anti rat  $\text{Fc}^{\text{E}}$  (20,000 cpm) was added to duplicates of the serum samples. The relationship of percent counts bound to the PCA titre for the two assays is shown (Figure 6).

LEGEND TO FIGURE 5

Duplicates of counts bound by doubling dilutions of positive serum in the PRASST. The effect of two concentrations of total added counts are shown.

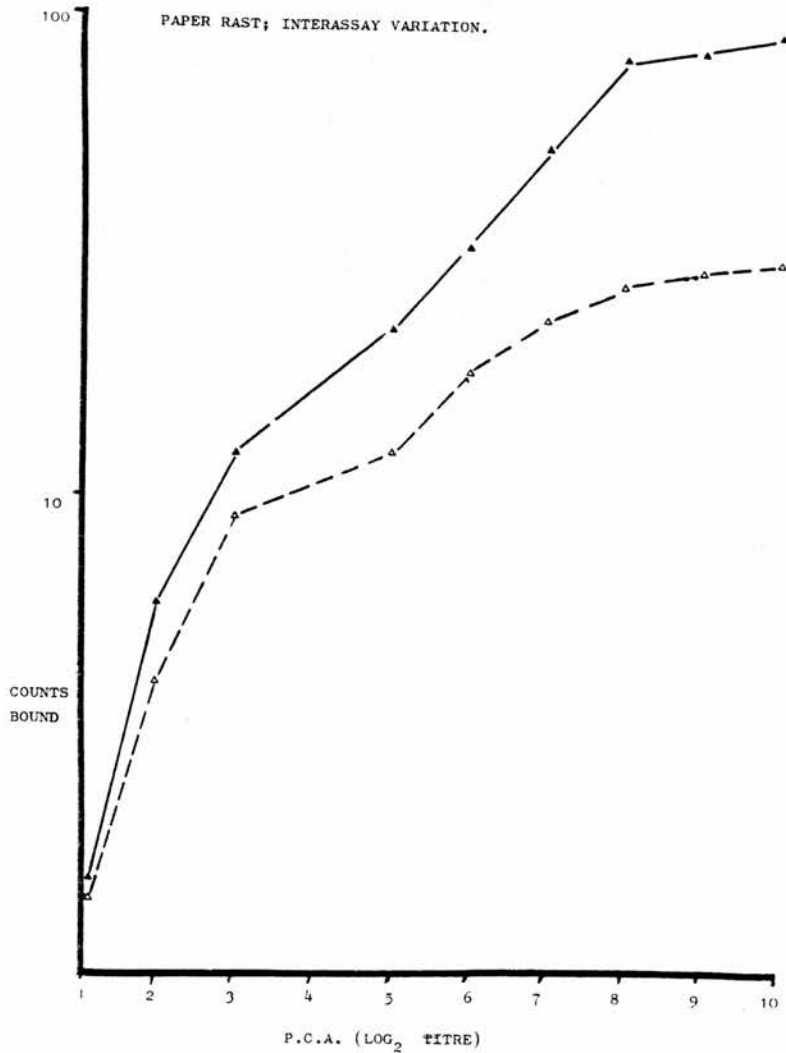


LEGEND TO FIGURE 6

Comparison of IgE antiovalbumin content of 9 sera tested by passive cutaneous anaphylaxis ( $-\log_2$  titre) and by PRAST (mean of duplicate assays, counts bound/counts bound by newborn serum) in separate batches.

The difference between samples measured in the two batches is significant. (Students t test for paired samples

$$t = 2.32 \quad p = 0.05 \quad n = 9)$$





The batches of ovalbumin-coated paper discs were prepared in an identical way using the same concentration of ovalbumin. Serum from unimmunized rats had  $< 1\%$  counts bound in both assays. However, counts bound by positive sera were significantly different in the two assays (Paired t test  $t = 2.35$   $p = 0.05$ ). Therefore comparison of IgE antiovalbumin values in the subsequent experiments was made only from results obtained in the same batch.

#### In Vitro Assay of IgE Anti $\beta$ lactoglobulin Antibody

$\beta$  lactoglobulin (Sigma Chemicals) was coupled to the paper discs as was ovalbumin (page 25). With two reference rat sera of known PCA titre a standard curve was obtained by the PRAST method and results expressed as counts bound/counts bound by neonatal rat serum.

#### PAPER RADIOIMMUNOSORBENT ASSAY FOR IgE.

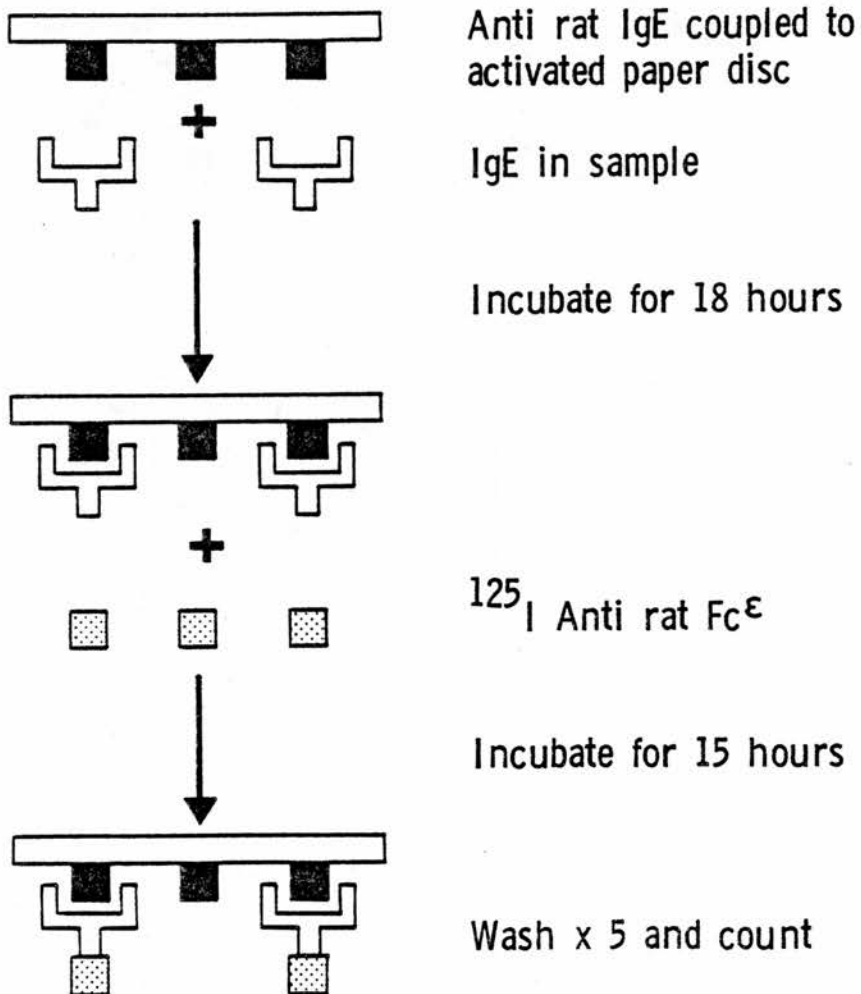
Serum IgE levels were measured by the paper radioimmunosorbent assay (PRIST) (Karlsson, Ellerson, Dahlbom, Bennich, 1979).<sup>(b)</sup> The method is outlined in Figure 7. Purified rabbit anti rat IgE antibody, kindly supplied by Dr. Jarrett, prepared and quantified by Dr. Karlsson, contained anti rat IgE in concentrations of 400  $\mu\text{g}/\text{ml}$ . 100  $\mu\text{g}$ s (250  $\mu\text{l}$ ) was coupled to 200 cyanogen bromide-activated discs in 20 mls of coupling buffer and treated similarly to the ovalbumin coated discs (page 25). 150  $\mu\text{l}$  of test serum in 100  $\mu\text{l}$  incubation buffer were added to each disc in plastic tubes and incubated on a rotating tray for eighteen hours. Unbound antibody was removed by washing 5 x in incubation buffer. 100  $\mu\text{l}$  of  $^{125}\text{I}$  anti rat  $\text{Fc}^{\text{E}}$  containing 150,000 cpm was added to each tube and incubated overnight. Surplus counts were removed by washing x 5 in 0.9% Saline and the tubes counted in a Wallac gamma counter.

A reference serum of known IgE concentration (0.7  $\mu\text{g}/\text{ml}$ ) and purified rat IgE myeloma protein (100  $\mu\text{g}/\text{ml}$ ) were kindly provided by

LEGEND TO FIGURE 7:

Scheme of the paper radioimmunosorbent assay  
(from Karlsson, 1978).

DIAGRAMMATIC SCHEME OF THE P.R.I.S.T

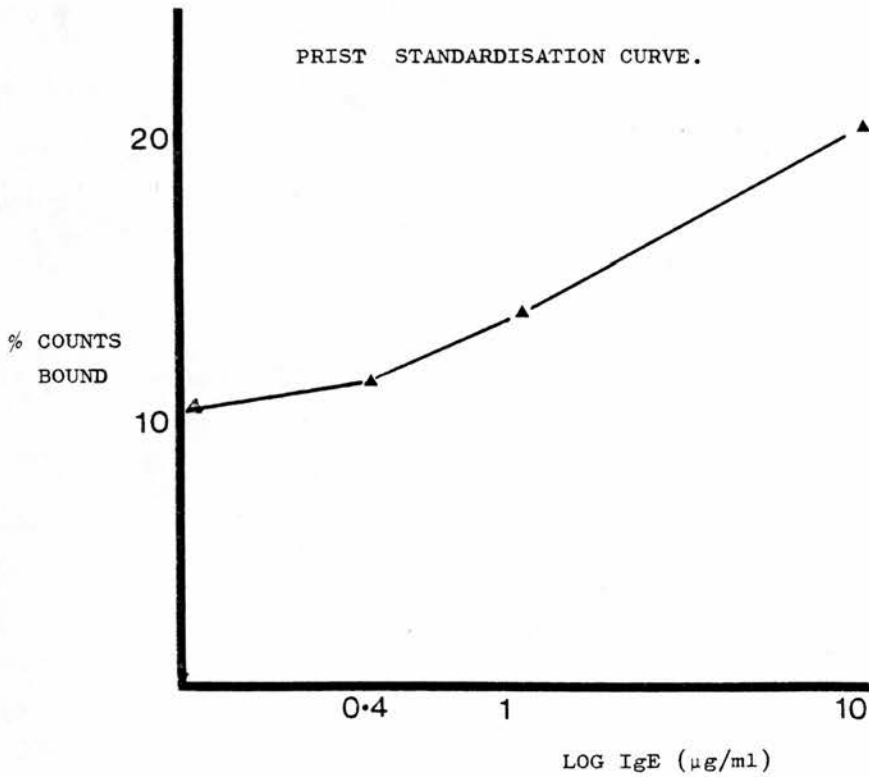


Dr. Jarrett. A standard curve was constructed from the assay of doubling dilutions of IgE myeloma at concentrations ranging from 1.0  $\mu\text{g/ml}$  to 15 ng/ml diluted in 10% unimmunized rat serum (Figure 8).

LEGEND TO FIGURE 8:

PRIST Standard curve:

Counts bound (percent of total counts) by known concentrations of rat IgE myeloma ( $\mu\text{g/ml}$ ).



Counts bound, multiplied by a dilution factor of 25, were plotted against IgE concentrations ( $\mu\text{g/ml}$ ). The sensitivity of the PRIST was impaired by non-specific binding of labelled antibody to the paper discs. Addition of different concentrations of  $^{125}\text{I}$  anti rat  $\text{Fc}^{\text{E}}$  (100,000 cpm and 50,000 cpm) to the tubes did not improve sensitivity of the assay, but it was sensitive enough for my purpose.

ASSAY OF ANTIOVALBUMIN AND ANTI  $\beta$  LACTOGLOBULIN ANTIBODY BY HAEMAGGLUTINATION.

Human erythrocytes (O-Rhesus negative) were coated with antigen by the method of Kofler et al. (1977). Fresh erythrocytes were washed 4 x in 0.15N NaCl, centrifuged, and resuspended in a 50% suspension in saline. 500  $\mu\text{l}$  of 50% erythrocytes were mixed with 2.5 mls. of 0.5% Ovalbumin (Sigma 5x crystallised) or  $\beta$  lactoglobulin in saline, and then mixed for five minutes with 0.1 ml. of 0.1% chromic chloride in saline. Antigen-coated erythrocytes were washed twice in phosphate buffered saline (PBS) pH 7.4 and suspended in a 1% solution in PBS. Test sera from immunized rats and positive reference sera were first absorbed with 50% washed unlabelled erythrocytes for thirty minutes. Doubling dilutions of absorbed serum in 50  $\mu\text{l}$  PBS were made on microtitre plates using Takatsi loops. 50  $\mu\text{l}$  of 1% labelled erythrocytes were added to each well; a control for non-specific agglutination was included by adding 1% unlabelled erythrocytes to the test serum. Human antiovalbumin and anti  $\beta$  lactoglobulin sera, kindly supplied by Dr. M.W. Turner, were used as positive controls. The plates were incubated at room temperature for sixty minutes and the haemagglutinating antibody titre ( $-\log_2$ ) was recorded.

To estimate IgG agglutinating antibody, doubling dilutions of absorbed serum were also made in 0.1M D.L. Dithiothreitol in PBS. (Olsen, Weiblen, O'Leary, Moscovitz, McCullough, 1976). Titres were reduced by  $> 2$  in only two of eighteen samples. Dithiothreitol treatment inactivates IgM immunoglobulin but it is possible that IgA contributed in a small degree to the agglutinating antibody titre.

#### OVALBUMIN CONTENT OF THE RAT DIET BY INHIBITION OF HAEMAGGLUTINATION

Free antigen will inhibit haemagglutination by positive serum (Stavitsky, 1964). A 1:5 saline extract of rat food (Oxoid breeder diet) and an aqueous filtrate of the milk supplement (Ch.3, page 39) were prepared.

Doubling dilutions of two positive sera were prepared in duplicate on microtitre plates. Agglutinating titres were 16 and 18 ( $-\log_2$ ) range of dilutions 2 to 24 ( $-\log_2$ ) 50  $\mu$ l of the rat food extract and of the milk filtrate were added to each well, followed by 50  $\mu$ l of 1% ovalbumin-coated erythrocytes. Agglutinating titres were not changed by this treatment.

#### PREPARATION OF RAT IgG ANTIOVALBUMIN ANTIBODY

Five mature HL rats were immunized with subcutaneous injections of 10 mgs. ovalbumin in Freund's complete adjuvant as previously described (page 19). Sera were tested for precipitin against ovalbumin on Ouchterlony plates (Ouchterlony, 1964). Dilutions of ovalbumin in concentrations of 2 mg/ml, 1 mg/ml, 0.5 mg/ml, 0.25 mg/ml, 0.125 mg/ml and 0.065 mg/ml were placed in wells in agar plates and allowed to diffuse against the test serum for twenty-four hours. Agar plates were inspected for precipitin lines, washed in saline, dried and stained with methylene blue. All sera were positive (Figure 9).

LEGEND TO FIGURE 9

Immunodiffusion analysis of serum from two rats immunized with ovalbumin in FCA: precipitin lines of the two sera against ovalbumin in concentrations ranging from 2 mg/ml to 0.065 mg/ml are shown. Excessive antibody concentration is shown.



The presence of IgG antibody in the immune serum was tested by immunodiffusion against sheep anti mouse IgG antibody (kindly provided by Dr.D. Stanworth) which cross reacts with rat IgG immunoglobulin. All rats were killed, exsanguinated and this pooled serum stored at  $-70^{\circ}\text{C}$ . Pooled serum was also obtained from five unimmunized rats; it did not have precipitin against ovalbumin.

Isolation of rat IgG containing Antiovalbumin Antibody by Ion Exchange Chromatography

A method similar to that of Karlsson(1978) was used. 15 mls. of pooled immune serum were equilibrated with 0.1M. Tris HCl buffer pH 8.0 by dialysis at  $4^{\circ}\text{C}$  for twenty-four hours. A column, 30 x 3 cms. filled with DEAE Sepharose CL6B was equilibrated with 0.1M Tris HCl buffer.

The pH and conductivity of the dialysed serum ( $8.0 \ 0.32 \times 10^3$ ) the eluting buffer ( $8.0 \ 0.23 \times 10^3$ ) and the eluate ( $8.0 \ 0.26 \times 10^3$ ) were checked before decanting the serum onto the column. Tris HCl perfused the column at a flow rate of 40 ml/hour. 15 ml. fractions were collected and the elution pattern determined by an LKB spectrophotometer at 280 nm. After elution of the first protein peak (fractions 12-20), absorbed proteins were eluted with buffer of increasing ionic gradient containing 0.7 M NaCl. Fractions containing the first peak were tested by immunodiffusion against ovalbumin (1 mg/ml) and anti mouse IgG serum. Fractions containing IgG antiovalbumin were concentrated by ultrafiltration to a final volume of 1.5 mls and protein concentration (Optical Density 10.2, Extinction coefficient 14) of 7.28 mg/ml and stored at  $-70^{\circ}\text{C}$ . This product consists of purified rat IgG immunoglobulin and includes anti-ovalbumin antibody. The preparation gave a single precipitin line when tested by immunoelectrophoresis against polyvalent antirat serum. (Behring reagents.)

#### STATISTICAL METHODS

Normally distributed data and log transformed data were analysed by students t test (two tailed distribution about the mean) for one variable (Zar, 1974).

Logarithmically distributed data were analysed by non-parametric methods; Mann Whitney U rank order test (Mann Whitney, 1947), Wilcoxon matched pairs test, and Fishers exact test for one variable.

Chi-squared analysis of contingency tables and Spearmans rank correlation coefficient were used for two variables.

### Chapter 3

#### A METHOD OF FEEDING RAT PUPS WITH SUPPLEMENTS OF A COWS MILK BASED PREPARATION

##### EXPERIMENTAL ANIMALS

Outbred Hooded Lister rats (HL) from two outbred colonies were used in the experiments. Most of the immunization procedures were performed with rats from the same colony as those used in previous experiments to investigate IgE antibody responses (Jarrett, Stewart, 1974) (Animal Suppliers Ltd., London), but the results were confirmed with HL rats from a different colony (Oxford Laboratory Animal Centre (OLAC), Bicester). One important difference between the two colonies was in control of cross infection. Using the Laboratory Animal Centre, Carshalton (Medical Research Council) classification, rats from Animal Suppliers Ltd. were category 1 and may have been exposed to parasitic infection within the breeding colony. Those from OLAC (Category 4) would not have been exposed to such infection. Differences between the HL colonies are discussed elsewhere (Chapter 8).

##### Experimental Groups

Investigation of IgE responses in several experiments were performed with rat pups of the same strain obtained from several litters. Groups of animals studied consisted of equal numbers from each of the litters so that inter-litter differences in IgE response did not influence the interpretation of results.

##### SUPPLEMENTARY COWS MILK AND SUPPLEMENTARY FEEDING

##### Preparation of Milk for the Supplementary Feeds

Based on the experience of others (Messer et al. 1969) (Hall, 1975) two cows milk-derived feeds were evaluated in the feeding experiments.



One preparation (Preparation A) consisted of four parts of evaporated cows milk diluted with one part of water. The second (Preparation B) was prepared from Cow and Gate premium infant feed, a dried cows milk formula (12g/100ml) enriched with casein (Casilian 7g/100ml) and 20% arachis oil (Prosparol) (see Appendix 1 for details). The chemical composition of these milks is shown in Table 1.

TABLE 1: CHEMICAL COMPOSITION OF RATS MILK AND THE COWS MILK BASED PREPARATIONS

Content	Rat Milk	Preparation A	Preparation B
Kilo Calories	159	158	160
Protein (g)	9.2 (73%Casein)	8.6	8.94
Lipid (g)	12.3	9.0	12.17
Carbohydrate (g)	3.0	11.3	3.76
Sodium (mg)	66	94	32
Potassium (mg)	140	241	75
Calcium (mg)	620	200	160
Phosphorus (mg)	229	163	30
Iron (mg)	0.61	0.61	0.5
Osmolarity (mosm/l)	352	670	620
pH	6.3 - 6.6	6.2	6.7

Preparation B more closely resembles rat milk in essential nutrients and contains similar amounts of the B vitamins and biotin, unlike Preparation A, consisting of diluted evaporated milk without vitamins.

A Method of Feeding Supplements of the Cows Milk Preparations By Intermittent Gavage.

Two methods have been described for feeding rat pups without suckling; repeated per oral gavage (Dymsza et al.1964) and continuous infusion of milk via gastrostomy (Hall, 1975). The former method was modified to provide supplementary feeds of the cows milk preparations described in Table 1; 3 cms. of narrow bore flexible silastic tubing (Portex, pp.10) internal diameter 0.28, mm, threaded onto a 25FG needle was used as a feeding tube, attached to a 1 ml syringe. It was

lubricated with vegetable oil and carefully inserted down the oesophagus into the stomach, assisted by reflex stimulation of sucking and swallowing. Correct positioning of the tube was confirmed by observation through the anterior abdominal wall of the milk bolus within the stomach.

Milk volumes were derived from observations of the degree of gastric distension after gavage. Feeding intervals were calculated from observations of milk intake in suckled rats (Morag, 1970) and shown in Table 2. Rat pups (Animal Suppliers Ltd.) were separated from their lactating mothers for twelve-hour periods each day and gavaged during this period according to the schedule shown in the Table.

TABLE 2: ESTIMATED DAILY MILK INTAKE AND CALCULATED FEEDING INTERVAL IN SUCKLING RATS FROM BIRTH TO WEANING

Age (Days)	Mean Weight (g)	Milk Volume /day mls	Milk Volume /feed mls	Feeds /day	Feeding interval hrs.
2	5	2.5	0.25	10	2.4
7	12	6.0	1.0	6	4
15	24	12.0	2.0	6	4
20	36	18.0	3.5	5	4.8

Mechanical complications of the feeding method were perforation of the oesophagus or stomach by the feeding tube and regurgitation and inhalation of the milk. These complications were encountered most frequently in the first three days of life and were invariably fatal. An additional problem of ophthalmia due to pasteurella, and infecting the whole rat colony is described elsewhere (Roberts, Gregory, 1980 ).

### Comparison of the Supplementary Feeds

Litters from two pregnant outbred Hooded Lister rats were separated from the lactating females for twelve hours daily, from the third to the tenth day of life.

Pups from litter 1 were gavaged with unsweetened diluted evaporated milk (Preparation A, Table 1). Normal suckling behaviour was resumed during the remainder of each twenty-four hour period. Pups from litter 2 were similarly gavaged with equal volumes of the preparation based on dried cows milk (Preparation B). The rat pups, supplementary fed with evaporated milk, died within ten days. Necropsy showed abdominal distension and milk curd obstruction of the stomach and small intestine. Rat pups supplemented with preparation B gained weight and had no evidence of disease. This preparation (supplement milk) was used in the subsequent feeding experiments.

### Feeding and Growth of Supplementary Feed Pups and Suckled Controls

Litters of Hooded Lister (HL) rat pups from three pregnant females were divided into unequal groups to compensate for anticipated fatalities in the supplemented group. One-third suckled continuously from the lactating females and were only removed for brief periods each day for measurements of body weight. Two-thirds were removed from the lactating female for twelve-hour periods each day and received supplementary feeds (Preparation D).

Continuously suckled control rats were marked by a subcutaneous injection of 0.1 ml. 1% Evans blue in saline. After growth of body hair at ten days of age, the animals were marked by cutaneous application of dilute picric acid. At weaning (twenty-one days) all animals in the litter were marked with incisions on the external ear. The numbers of rats in each group at weaning were similar.

Fifteen rat pups receiving supplementary feeds were placed in beakers in a water bath of constant temperature (37°C) and a relative humidity of 60%. They received supplementary milk by repeated gavage in volumes equal to half their expected nutritional and fluid requirement (Table 2, page 39). Five died as a result of trauma from the gavage procedure between birth and ten days of age. After growth of body hair the pups were placed in straw-lined cages and drank a dilute preparation of Cow and Gate premium feed and libitum from a water dropper, in addition to the supplementary feeding by gavage. The mean weight gain of ten supplementary feed rats was greater than eleven controls (Figure 10). One group of six pups receiving only the artificial feed throughout each twenty-four hours gained little weight and died.

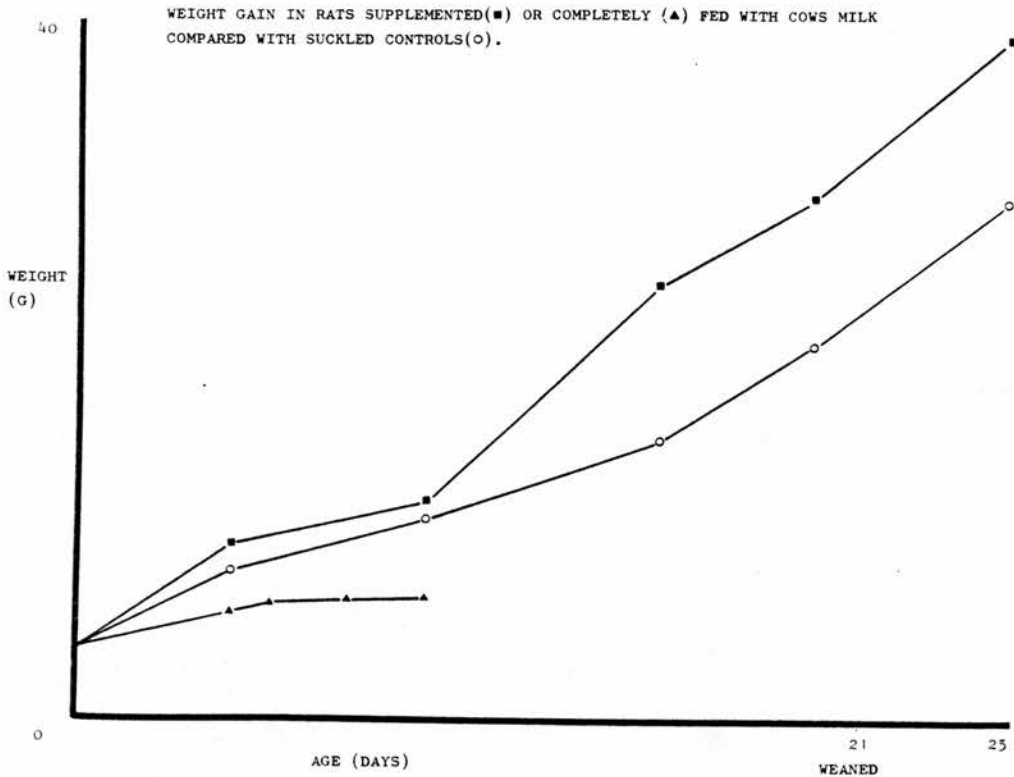
FIGURE 10. MEAN WEIGHT OF RAT PUPS RECEIVING  
THREE FEEDING REGIMES

LEGEND: The mean weights of three groups of rats from three litters of HL rats are shown.

(■)

Ten received supplements of the cows milk-based preparation in addition to maternal milk. Six (▲) received the cows milk preparation and no maternal milk. Eleven (O) received maternal milk and no supplementary feed. Ingested milk volumes were similar in the three groups. All rats fed only with the cows milk preparation died by ten days of age.

FIGURE 10:



#### Effect of type of Milk on Intestinal Growth

One litter of twelve HL rat pups were divided into three equal groups at birth. Four animals were immediately killed before the onset of suckling; the intestine from proximal duodenum to rectum was removed, washed in saline and weighed. The remaining animals were fed for twenty-four hours and then killed; one group suckled normally and one group received supplement milk by gavage in volumes of  $0.5 \text{ ml}^{-1} \text{ g}^{-1} \text{ body weight day}^{-1}$ .

The weight of the intestines of the suckled rat pups was significantly greater than the ones receiving the cows milk preparation (Table 3, p. 43).  $t = 4.48$   $p < 0.005$ .

Some of the increase in weight in the first day of life may be explained by a residue of unabsorbed milk in the intestines but this could not account for the whole difference between the rats fed with the different milks.

TABLE 3:    INTESTINAL WEIGHT (MEAN AND S.D.) OF RAT PUPS AT BIRTH  
              AND AFTER FEEDING WITH DIFFERENT MILKS FOR TWENTY-FOUR  
              HOURS

	n	Mean (g) Weight	S.D.	% Increase
Birth	4	1.41	0.136	—
Suckled	4	2.13	0.219	51.1
Fed with the cows milk-based preparation	4	1.64	0.191	16.3

#### Discussion of the Feeding Experiments

Unlike human infants, rat pups fed entirely with cows milk preparations fare badly; in these experiments they gained weight slowly in comparison with suckled litter mates and died by day ten. There have been only two reports of successful rearing with artificial feeds (Messer et al. 1969) (Hall, 1976) requiring sophisticated infusion devices and gastrostomy tubes. The relatively poor weight gain of these animals may have been related to low milk intakes (approximately 60% of the volumes shown in Table 2), but other factors such as infection and malabsorption probably contributed to the failure to thrive. Rat pups reared in this way were considered unsuitable for comparison with suckled litter mates because many variables other than diet may have contributed to subsequent IgE responses. In contrast, supplementary feeds derived from milk, and administered by intermittent gavage, resulted in satisfactory growth rates in rat pups who received some rat milk by suckling. Two supplementary milks were evaluated, derived from dried cows milk enriched with fat emulsion and casein, and an unsweetened evaporated cows milk of similar caloric content. The first of these preparations appeared to be better digested and absorbed and was therefore used in subsequent experiments to determine the effects of

supplementary feeds on the IgE response. It is possible that some effects of the experimental manipulation were produced by the gavage procedure rather than the dietary content. Such effects would be of significance in interpretation of the effects of cows milk-based supplementary feeds; they may have been inhaled in small amounts into the trachea, resulting in pulmonary sensitization and an IgE response to cows milk proteins. A similar mechanism of sensitization has been observed in guinea pigs (Anderson et al. 1979). In addition, orogastric intubation may have introduced infection into the gastrointestinal tract affecting the animals response to subsequent immunization. The ideal control animal, that of rat pups gavaged with rat milk, was not possible because lactating females could not be milked in sufficient quantities to provide adequate nourishment for the pups. Control animals were therefore not gavaged and suckled normally until weaning. The practise of feeding newborn human infants with tube feeds of cows milk preparations is common. I know of no clinical experience that this results in greater sensitization than that in bottle-fed infants.

Another advantage of the supplementary fed animals is that it receives some colostrum, one effect of which is to promote normal intestinal growth. Newborn rats fed entirely with a cows milk preparation had smaller intestines than suckled rats, suggesting that mucosal absorption including that of antigenic material may become significantly different in artificially fed rats. Malnourished human infants show several immunodeficiencies (Mathews et al. 1972); as do protein deprived mice (Passwell et al. 1974), so the malnourished artificially fed rat is therefore unsuitable for comparative studies of immune responses.

These experiments have demonstrated the dependence of suckling rats on maternal milk. Supplementation of this diet with a cows milk-based preparation permits satisfactory growth, as in human infants, but adverse immunological effects of cows milk-based diets to suckling rats have now been demonstrated.

## Chapter 4

### FACTORS INFLUENCING THE IgE RESPONSE IN ADULT AND YOUNG RATS

#### INTRODUCTION

Most of the experiments described in this chapter replicate previously reported studies. The IgE antiovalbumin response in immunized rats compared with unimmunized controls, was validated by comparison of the results in the PRAST with those obtained by P.C.A. The effect of antigen dose and route of administration on the IgE responses, previously reported by Jarrett et al. (1974, 1976), were studied using the same HL rat strain. Pauwells et al. (1979) have shown that young rats produce a greater IgE response than adult ones; this, too, was confirmed.

The effect of neonatal antigen contact on the IgE response have not previously been investigated, so I studied the effects of antigen dose and route of administration on IgE responses in rats immunized at weaning.

#### CORRELATION OF PAPERS RADIOABSORBENT TEST AND PASSIVE CUTANEOUS ANAPHYLAXIS.

Hooded Lister rats from six litters were immunized at weaning (twenty-two days) with intraperitoneal ovalbumin and pertussis adjuvant (Chapter 2, page 20). Both ovalbumin doses 10 µgs and 1 µg, given in the first and second injections respectively, were similar to those given to adult HL rats in previously reported studies (Jarrett et al. 1974). Serum from sixty-day old rats was tested in duplicate by PRAST. IgE antiovalbumin results, expressed as counts bound/counts bound by newborn rat serum, ranged from 1-90. Newborn serum bound <1% of the total counts added. Sera from fourteen of the rats with different IgE anti-ovalbumin values were then tested by passive cutaneous anaphylaxis



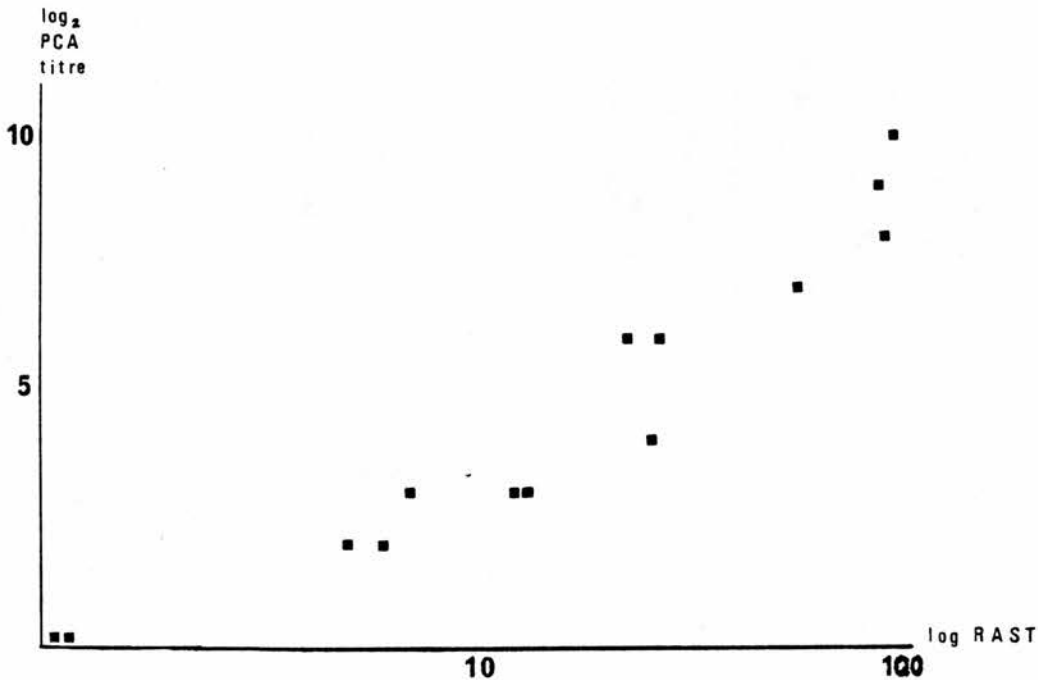
(Chapter 2, page 21); the results ( $-\log_2$  titre) correlated significantly with the PRAST values. Spearman's rank correlation coefficient  $r = 0.9985$   $p < 0.001$  (Figure 11).

Since IgE antibody was observed in the presence of IgG antibody the latter did not prevent detection of IgE antibody by PCA or PRAST.

LEGEND TO FIGURE 11:

IgE antiovalbumin antibody assayed in 14 sera by PRAST (counts bound/counts bound by newborn serum) and passive cutaneous anaphylaxis ( $-\log_2$  titre). They correlate significantly  $r = 0.9985$   $p < 0.001$ .

Correlation of IgE antiovalbumin by in vivo and in vitro methods



EFFECT OF ANTIGEN DOSE AND ROUTE OF ADMINISTRATION ON  
IgE RESPONSE IN ADULT RATS

Injected Antigen: Fifty adult male HL rats (body weight 250-350g) were purchased from Animal Suppliers Ltd. They were caged in groups of four or five and fed dried food (Oxoid breeder diet) and water and libitum. The diet did not contain ovalbumin (Chapter 2, page 34). Eight rats received 10 µgs intraperitoneal ovalbumin with  $10^{10}$  heat-killed Bordatella pertussis followed by a second intraperitoneal injection of 1.0 µg ovalbumin without pertussis fourteen days later. Nine rats received 1000 µgs of ovalbumin with pertussis, and a second injection of 1.0 µgs ovalbumin. The IgE antiovalbumin response (counts bound/counts bound by newborn serum) was significantly less in the rats immunized with the higher dose (Figure 12); in six of them, no antibody was detected. (Low and high dose groups. Mann Whitney U= 9 p= 0.005.)

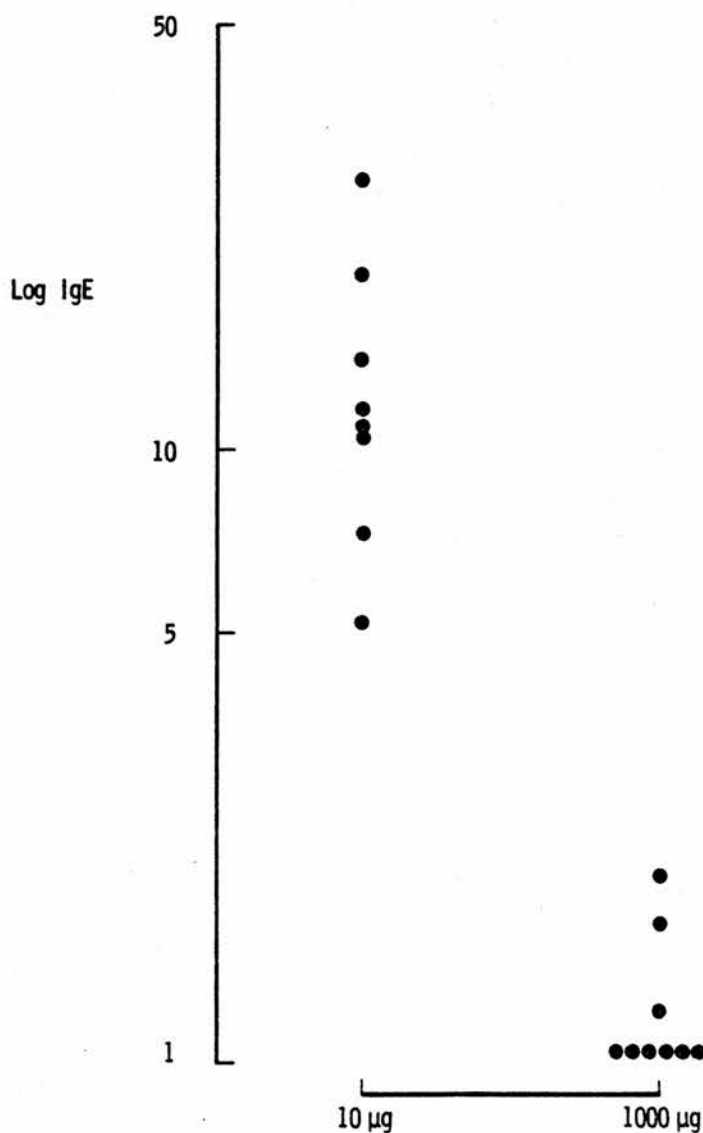
Oral Antigen: Thirteen animals were fasted and then gavaged with ovalbumin in doses of 10 µgs (seven rats) or 1000 µgs (six rats) and simultaneously injected intraperitoneally with  $10^{10}$  Bordatella pertussis. All animals received a second immunization with 1.0 µg oral ovalbumin, without adjuvant, fourteen days later.

Five of the six animals orally immunized with the higher antigen dose had IgE antiovalbumin values  $\leq$  newborn rat serum. The response in seven animals immunized with the low dose was considerably less than in rats immunized with the same dose by intraperitoneal immunization (Mean 1.77 range 1.45-2.96). The IgE antiovalbumin response to oral antigen was less than that reported by Jarrett et al. (1976) though the antigen dose and the route of adjuvant administration were identical. The effect of other doses of oral antigen was not explored.

LEGEND TO FIGURE 12:

Secondary IgE antiovalbumin antibody (counts bound/  
counts bound by newborn serum) in mature Hooded Lister  
rats immunized by intraperitoneal injection with 10 µgs  
or 1000 µgs ovalbumin and adjuvant followed by 1.0 µg  
of ovalbumin alone (see text). The antigen dose effect  
is significant. (Mann Whitney U= 9 p= 0.005.)

### Effect of high and low antigen dose on IgE response in adult rats



Sera from eighteen unimmunized adult rats were tested by PRAST in the same batch as the immunized animals. The mean value was 1.1, range 0.8 - 1.4 (count bound/counts bound by newborn serum). The samples were pooled and tested by P.C.A.; IgE antiovalbumin was not detected by this method. It is likely that values  $< 1.5$  represent non-specific binding of counts in the PRAST and that unimmunized rats have no IgE antiovalbumin antibody.

#### IgE RESPONSES IN YOUNG HL RATS

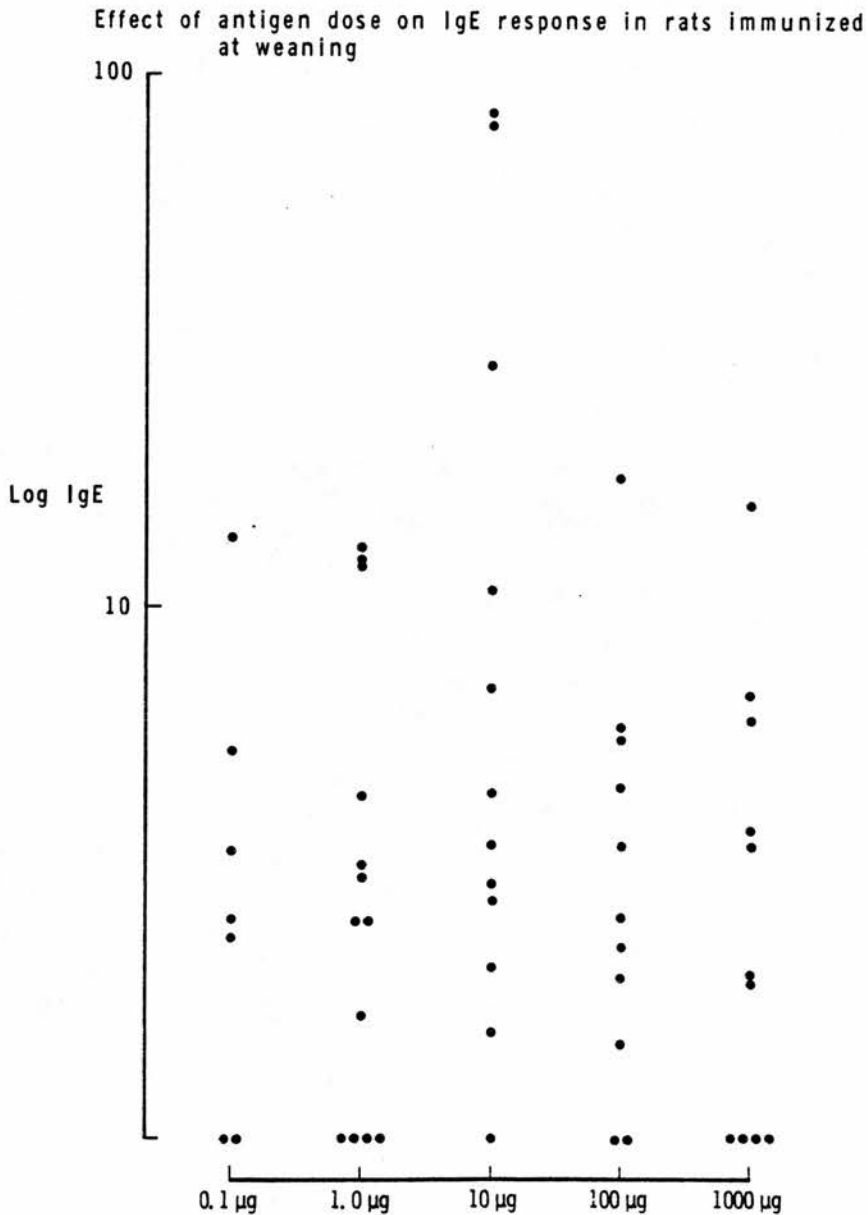
##### (i) Optimum antigen dose in rats immunized at weaning.

Litters from six pregnant HL rats (Animal Suppliers Ltd.) were divided into five groups at weaning age (twenty-one days). The groups contained rats from all the litters to eliminate the influence of genetic bias in antibody responsiveness. They were immunized by intraperitoneal injections with ovalbumin in doses of 0.1, 1.0, 10, 100 and 1000  $\mu$ gs. with  $10^{10}$  Bordetella pertussis on the 22nd day of life, and with 1.0  $\mu$ g ovalbumin on day 56, and were bled four days later. There were no significant differences in the IgE antiovalbumin responses to different antigen doses (Figure 13) unlike the findings in adult rats (page 48) but the highest response was to 10  $\mu$ gs ovalbumin (Median 4.0. range 1-84). IgE antiovalbumin was detected in 7/11 of these young rats immunized with 1000  $\mu$ gs. OA, compared with 2/9 adult rats similarly immunized. (Fisher Exact Test  $p = 0.05$ .)

The response to high antigen dose in young and adult rats appeared to be different, but the groups are not strictly comparable because the antibodies were assayed in different batches.

LEGEND TO FIGURE 13:

Secondary IgE antiovalbumin antibody (counts bound/  
counts bound by newborn rat serum) in rats immunized at  
weaning by intraperitoneal injection of 0.1, 1.0, 10, 100  
and 1000  $\mu$ gs. ovalbumin with adjuvant, as described in  
the method. Highest median value is in the group immunized  
with 10  $\mu$ gs (4.0). Differences between the groups are  
not significant.



(ii) Primary and Secondary IgE Responses.

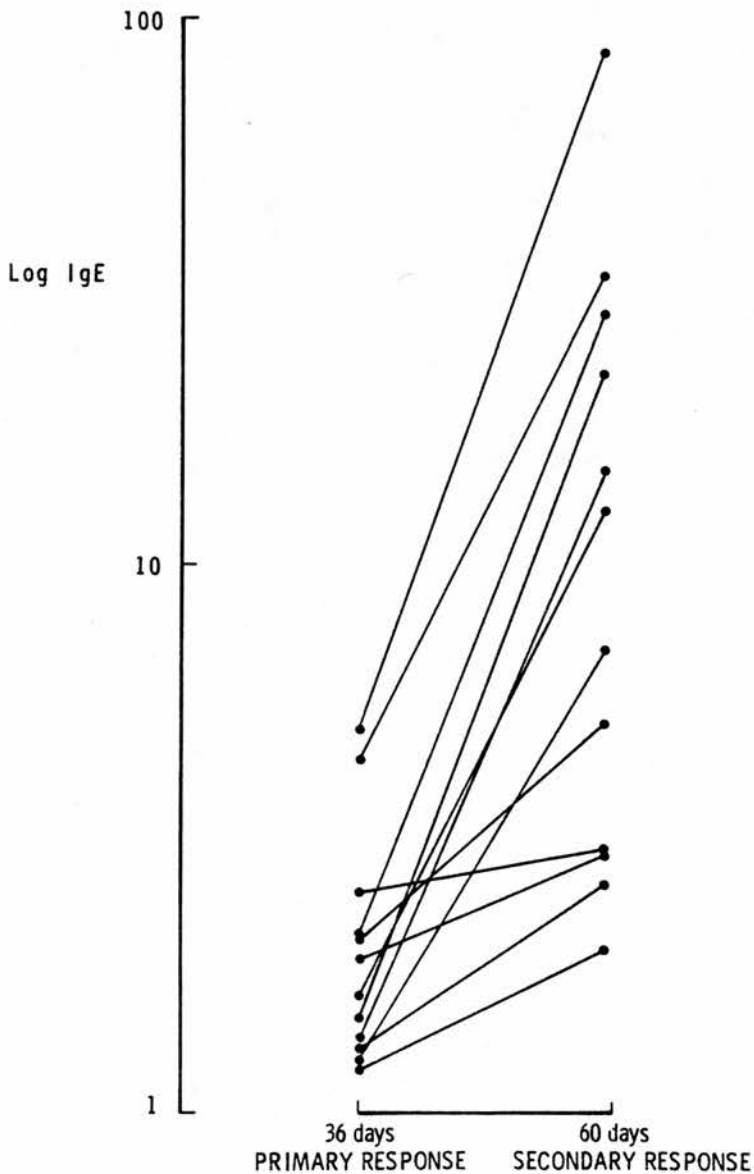
Twelve HL rat pups from one litter were weaned and immunized with 10 ugs. intraperitoneal ovalbumin and pertussis adjuvant on day 22. Nine pups of the same age from another litter were unimmunized controls. 1.0 ml blood was obtained from the tail vein at thirty-six days of age, fourteen days after the primary injection. Immunized rats received 1.0  $\mu$ gs ovalbumin on day 56 and blood withdrawn by cardiac puncture four days later. Paired sera were tested by PRAST and IgE antiovalbumin values, in the twelve immunized rats are shown in Figure 14. No response was detected at thirty-six or sixty days in unimmunized rats. The difference between primary and secondary responses was highly significant (Wilcoxon matched pairs rank test.  $p. < 0.001$ ). The highest values in the primary response were observed to give the greatest secondary response (Figure 14). In subsequent experiments, effects were studied only on the secondary responses.



LEGEND TO FIGURE 14:

Primary and secondary IgE antiovalbumin response is shown in rats immunized at weaning by intraperitoneal injection of 10  $\mu$ gs ovalbumin with adjuvant and with 1.0  $\mu$ g ovalbumin on day 56. The difference between the primary and secondary response is significant (Wilcoxon matched pairs rank order test  $p < 0.001$ ).

IgE Antiovalbumin response after the first and second injections of Ovalbumin



(iii) The IgE Responses to Oral Antigen in Suckling Rats

A group of 6 ten-day old pups from one litter were injected intraperitoneally with 10 ugs ovalbumin and pertussis adjuvant. The animals died within six hours of injection and no further experiments were performed in animals of this age group.

Fifteen pups from two litters were studied at the age of seventeen days. They were fasted for four hours and then gavaged with 10  $\mu$ gs ovalbumin and  $10^{10}$  Bordatella pertussis. They continued to suckle until twenty-one days and were then weaned. None died. Ten pups from one litter were similarly immunized after weaning with oral ovalbumin on day 22. The twenty-five animals in both groups received 1.0  $\mu$ g oral ovalbumin on day 56 and were bled four days later. IgE antibody was detected in only two of fifteen rats immunized before weaning, and in one of ten immunized after weaning. Antibody values in the two immunized before weaning (1.58 and 1.9) and the one immunized after weaning (1.57 counts bound/counts bound by newborn serum) were extremely low. The failure of oral antigen to sensitize young rats was similar to that observed in adult rats and was not influenced by administration of antigen before or after weaning.

(iv) The Effect of Age on the IgE Response

Eighteen HL rat pups (weight 28-36g), twelve adult HL rats (weight 242-312g) and seven weaning Wistar rats (weight 51-67g) were immunized with intraperitoneal ovalbumin (0.2  $\mu$ g/g. Body Weight) and  $10^{10}$  Bordatella pertussis. A second injection of 1.0  $\mu$ g ovalbumin was given to all groups twenty days after the first injection. The IgE antiovalbumin, assayed in the same batch, is shown in Figure 15. The greater response in the weaning HL rat pups than in adults was confirmed. (Mann Whitney U= 61 p= 0.05.) Strain related differences in rats



immunized at weaning was also demonstrated since the HL group gave significantly higher responses than the Wistar group (Mann Whitney  $U = 26$   $p < 0.05$ ).

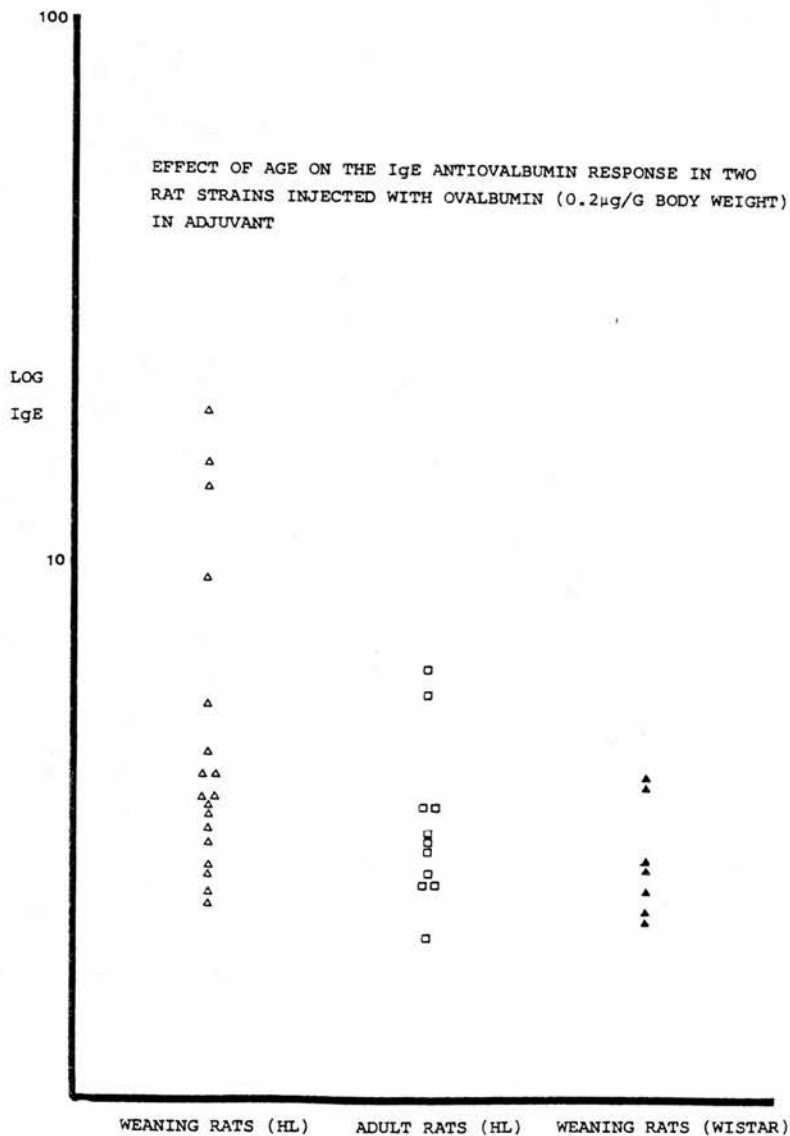
The variables of age and antigen dose were independently investigated. Adult and young HL rats were immunized either with 10  $\mu$ gs ovalbumin or 0.2  $\mu$ g ovalbumin/g Body Weight in the primary injection. All the rats received 1.0  $\mu$ g ovalbumin in the second injection because the magnitude of the secondary response depends on the antigen dose in the first injection (Jarrett et al. 1974). The secondary IgE responses, shown in Figure 16, are not different for the two dose regimes, and show in each case the greater responsiveness of the young rats. (Mann Whitney  $U$  (10  $\mu$ gs) 54  $p < 0.05$ . MWU (0.2  $\mu$ g/g) 5  $p = 0.005$ .)

### Discussion

The radioallergosorbent test for IgE antiovalbumin antibody correlated strongly with the *in vivo* assay. The sensitivity of the assays was similar but the RAST discriminated between low low antibody values better than PCA because values between 1 and 15 (counts bound/counts bound by newborn serum) were confined to titres ranging from 0 to 3 ( $-\log_2$ ). The presence of IgG antiovalbumin in the serum samples did not prevent detection of the IgE antibody either by RAST or PCA, and this has been confirmed by others (Karlsson, 1978) (Jarrett et al. 1974). Non-specific binding of the radiolabel of  $< 1.5\%$  of total added counts was similar to that bound by newborn rat serum which contains no IgE immunoglobulin, confirming the validity of the RAST assay (Karlsson, 1978). The animals did not receive ovalbumin in the rat diet and did not produce IgE antiovalbumin antibody without active immunization. Batches of results differed in each RAST assay. Variables such as preparation of the discs,

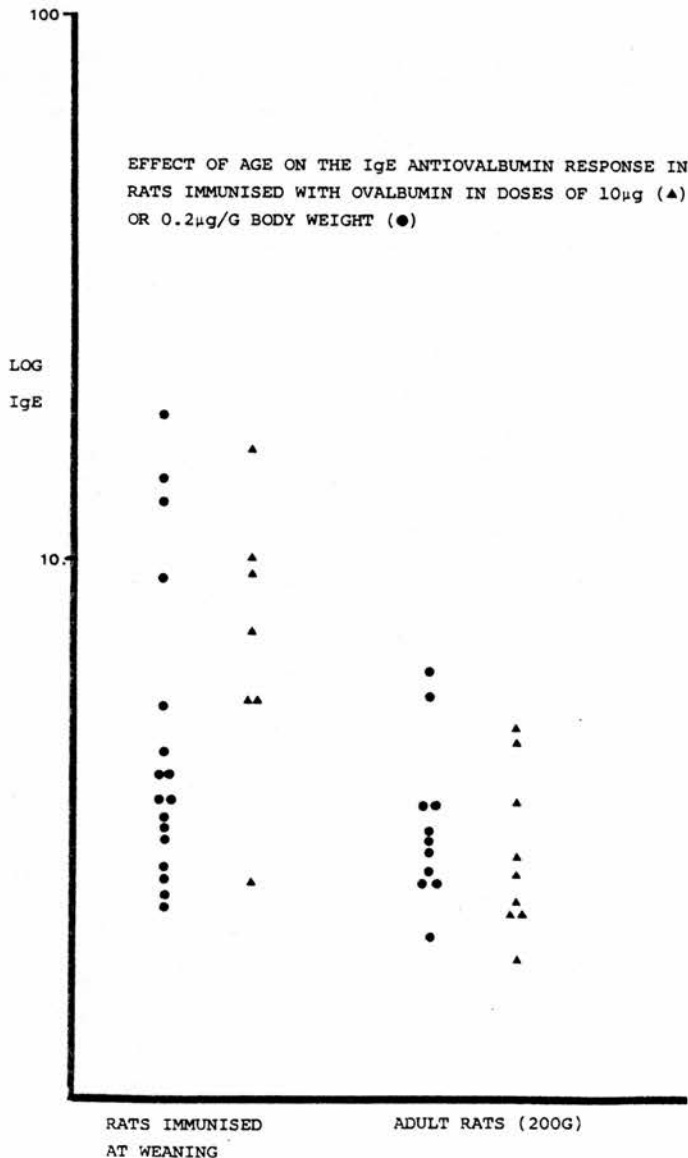
LEGEND TO FIGURE 15:

Secondary IgE antiovalbumin response in three groups of rats immunized by intraperitoneal injection of 0.2  $\mu$ g/g body weight ovalbumin with pertussis adjuvant. The difference between adult HL rats and HL rats immunized at weaning is significant. (Mann Whitney U = 26 p < 0.05.) Median values for the three groups are 3.6, 3.0 and 2.6 (counts bound relative to binding by newborn serum) respectively.



LEGEND TO FIGURE 16 :

Secondary IgE antiovalbumin response in rats immunized at weaning and adult rats, injected with ovalbumin in doses of 10 ugs(▲) or 0.2 µg/g body weight (●) in the primary injection. The difference between the young and adult rats is significant for both antigen dose regimens (Mann Whitney U =54  $p < 0.05$  and MWU= 5  $p = 0.005$ ).



length of incubation and amount of labelled antibody have been shown to influence this interassay difference (Karlsson, Ellerson, Haig, Jarrett and Bennich, 1979.)<sup>(a)</sup> For this reason all samples in each experiment were assayed in the same batch. I confirmed that adult HL rats readily produce IgE antibody when injected with small doses of antigen and adjuvant. Larger doses suppress the response. Unlike previous experiments, the response to low doses of oral antigen was considerably less than that with injected antigen; the explanation for this poor response is uncertain. The dose of the second antigen administered may have contributed since Jarrett et al. (1976) showed the size of the response was greater with a second antigen dose of 100 µgs ovalbumin than with 1.0 µg used in my experiments. The magnitude of the secondary response depends on the size of the first dose of injected antigen but this may not be true for ingested antigen. The effect of increasing the second dose of oral antigen was not explored. Using a different rat strain, Bazin and Platteau (1976) found that much larger ovalbumin doses were most effective (50-100 mgs) and that immunization by the oral route did not reliably produce an IgE response.

The response to injected antigen early in life has not been extensively studied; it differs in several respects from that in adult HL rats. The optimum antigen dose for IgE response in rats immunized at weaning was 10 µgs ovalbumin but the response to higher doses (1000 µgs) did not show the degree of suppression seen in adult rats. It has been suggested that such large doses stimulate T suppressor lymphocytes (Tada, 1975) (Ishizaka, Adachi, 1976) (Jarrett, 1977); this mechanism may be immature in young rats. The observation may be of significance for IgE responses in human infants since there is some evidence that T-cell populations are deficient at this age, particularly in atopic infants (Juto, Stannegård, 1979). The higher IgE response in young rats

reported by Pauwells et al. (1979), is confirmed by my experiments. With both antigen dose regimes young rats produced significantly more antibody than comparably immunized adult rats. This observation may result from immaturity of suppressor mechanisms, particularly in young HL rats; they had a significantly greater response than Wistar rats of the same age.

Antigen administered by stomach tube, together with injected adjuvant failed to produce an IgE response in young rats. Antigen dose was similar to that used in adult rats so the lack of response was probably the result of ineffective immunization. Antigen absorption is probably greater before weaning (Rodewald, 1973)(Abrahamson et al.1979) but attempts to immunize seventeen-day old rats with oral antigen were also ineffective. It is probable that the antigen doses in the first and second immunization were inappropriate but an additional possibility is that they produced systemic tolerance. Systemically injected animals fail to produce antibody if pretreated with a single oral dose of antigen early in life (Thomas,Parrott, 1974)(Swarbrick, Stokes and Soothill,1979). However tolerance is not induced in experimental animals when antigen is given orally with adjuvant, and IgE responses in rats have been observed whether adjuvant was administered by mouth or intraperitoneal routes (Bazin and Platteau, 1976)(Jarrett et al. 1976).

In conclusion the antigen dose-dependant IgE response reported by Jarrett in adult rats, when injected with antigen and adjuvant, has been confirmed. Young rats of the same strain produce a greater response and respond to a wider range of antigen doses than adults. The IgE response to oral antigen in young and adult rats was unpredictable; in many animals no response was detected and in others the response was of small magnitude, so immunization with injected antigen was preferred in my subsequent experiments.

## Chapter 5

### SUPPRESSION OF IgE RESPONSE IN YOUNG RATS BY MATERNAL ANTIBODY

#### INTRODUCTION

Young HL rats readily produce an IgE response and this is not suppressed by high antigen dose, unlike adult rats. That antigen-specific suppression was found in the young offspring of immunized females (Jarrett, Hall, 1979) is of considerable interest. Cross-fostering experiments demonstrated that the effect was dependent on milk from the immunized lactating females. It is possible that the observation has relevance to the breast-fed infants of atopic parents, but such an antigen-specific suppression has not yet been shown in human infants. Unlike breast-fed infants, the suckled newborn rat absorbs maternal IgG immunoglobulin from the gastrointestinal tract (Rodewald, 1973) (Borthistle et al. 1977); it is possible that such antibody influences the IgE response to immunization in suckled offspring by feedback regulation (Tada, Okumura, 1970). Therefore, the effect of passively administered IgG antiovalbumin antibody on IgE antiovalbumin response was investigated in the young rat.

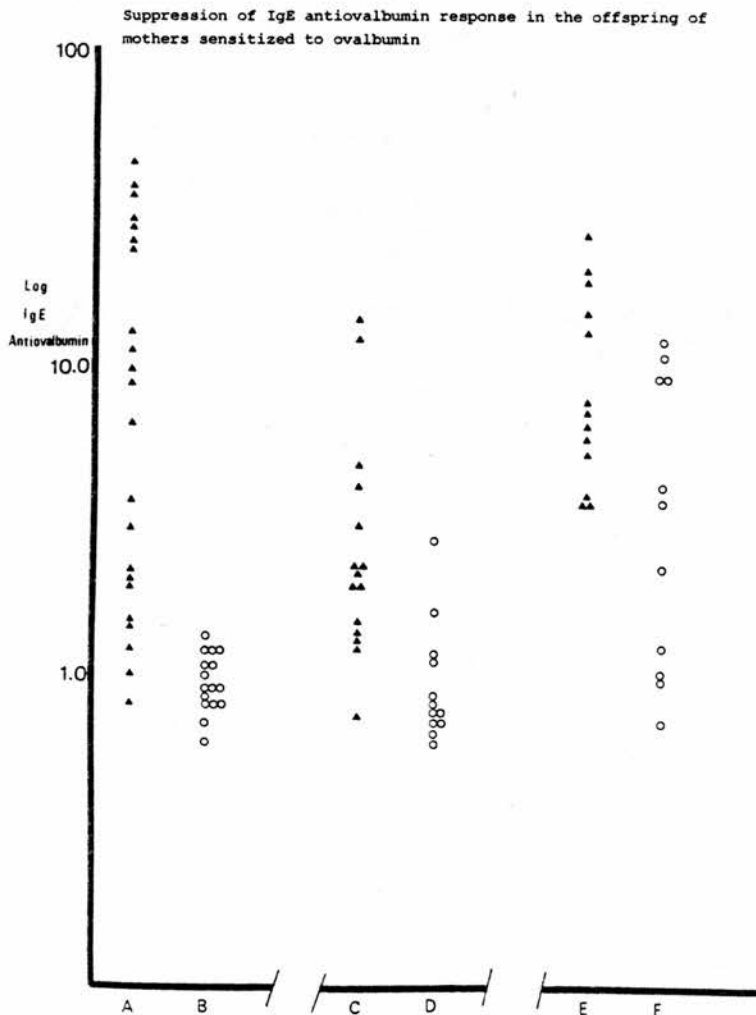
#### SUPPRESSION OF IgE RESPONSE BY MATERNAL INFLUENCE

Ten female HL rats (Animal Suppliers Ltd.) were immunized with a single intraperitoneal injection of 10 mgs. ovalbumin with  $10^{10}$  Bordetella pertussis. Serum obtained two weeks after injection contained agglutinating antibody to ovalbumin (range of titres 14-18). The ten immunized females and ten unimmunized females were mated and produced twelve litters within five weeks of immunization. Two immunized and two unimmunized females suckled their offspring. Litters from four unimmunized females were cross-fostered within twelve hours of birth

with four immunized females, thereby receiving a negligible amount of maternal milk. The pups were weaned at twenty-one days of age and injected intraperitoneally with 1.0  $\mu$ g ovalbumin and  $10^{10}$  Bordatella pertussis on day 22. The secondary IgE responses in the different litters are shown in Figure 17.

LEGEND TO FIGURE 17:

IgE antiovalbumin values (counts bound/counts bound by newborn serum) are shown in six groups of rats from twelve litters. Groups, A, C and E suckled from unimmunized females; Groups B,D and F suckled from females immunized with ovalbumin. C and E were crossed from immunized mothers who fostered pups in groups D and F.



Suppression of IgE response in the offspring of immunized females (Group B) was shown; the response was significantly less than that in the offspring of unimmunized females (Group A). (Mann Whitney  $U = 21$   $p < 0.001$ .) The difference between the cross fostered litters confirmed the effect of milk in suppressing the IgE response. (Mann Whitney  $U$  (C and D) 20.5  $p < 0.01$  and (E and F) 37  $p < 0.05$ .) Though the suppression was significant, it was not to the same degree as the difference between the uncrossed litters.

SUPPRESSION OF IgE ANTIOVALBUMIN RESPONSE BY INGESTED SERUM CONTAINING ANTIOVALBUMIN ANTIBODY

Six HL rats were immunized with three subcutaneous injections of 10 mgs. ovalbumin in Freund's complete adjuvant (Chapter 2, page 19). Serum obtained from the rats was tested for antiovalbumin antibody by immunodiffusion; immune serum was pooled and diluted 1:8 in normal saline so that the immunoglobulin concentration approximated to that of rat milk (McGhee, Michalek, Ghanta, 1975).

Litters from two unimmunized HL females were divided equally into two groups. One group received 0.5 ml of the diluted immune serum by stomach tube each day between the ages of five and twenty-one days. The control group received an equal volume of dilute serum from unimmunized adult rats. The secondary IgE response in the two groups is shown in Figure 18.

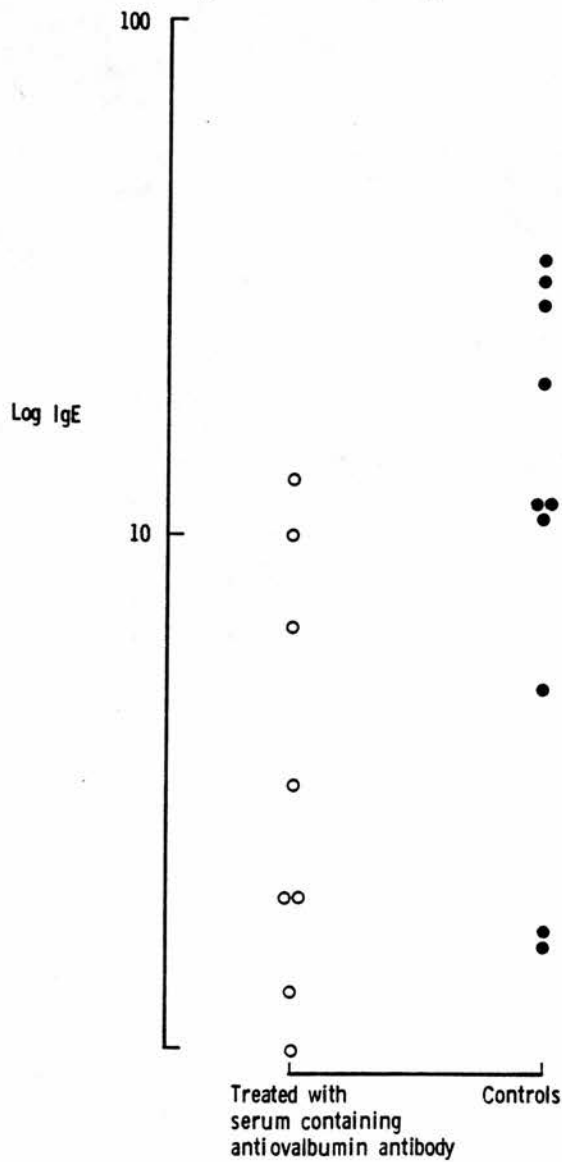
The response in the rats treated with serum containing antiovalbumin antibody was significantly less than the controls. (Mann Whitney  $U = 8$   $p = 0.005$ ). The degree of suppression is comparable with that observed in the rats who received milk from immunized females (Figure 17).



LEGEND TO FIGURE 18:

Secondary IgE antiovalbumin response in rats gavaged with serum containing antiovalbumin antibody and in litter mate controls. Suppression of IgE response in the treated group is significant (Mann, Whitney  $U = 8$   $p = 0.005$ )

Suppression of IgE antiovalbumin response by oral administration of serum containing antiovalbumin antibody



### Effect of Oral IgG Antibody on the IgE Response

Serum from rats immunized with 10 mgs. ovalbumin in Freund's complete adjuvant was fractionated by ion exchange chromatography (Chapter 2, page 35). The IgG fraction so obtained was diluted 1:20 in normal saline; it contained precipitating antibody to ovalbumin and a protein concentration of 0.36 mg IgG/ml.

One litter of fifteen HL rat pups was divided into two groups. Six were gavaged with 0.5 ml, of the IgG preparation each day between the ages of five and fifteen days. Nine were not gavaged and suckled normally. At weaning on day 22, the rats who had received the IgG preparation and six control animals were immunized with intraperitoneal ovalbumin (1.0 µg) and pertussis adjuvant. The secondary IgE response is shown in Figure 19 and compared with the values in three controls, also from the same litter, who were not immunized. IgE antiovalbumin was detected in both groups of immunized rats but not in unimmunized controls. The response was less in those animals treated with the IgG preparation and this approached significance (Mann Whitney U = 8 p = 0.06) when analysed by non-parametric methods.

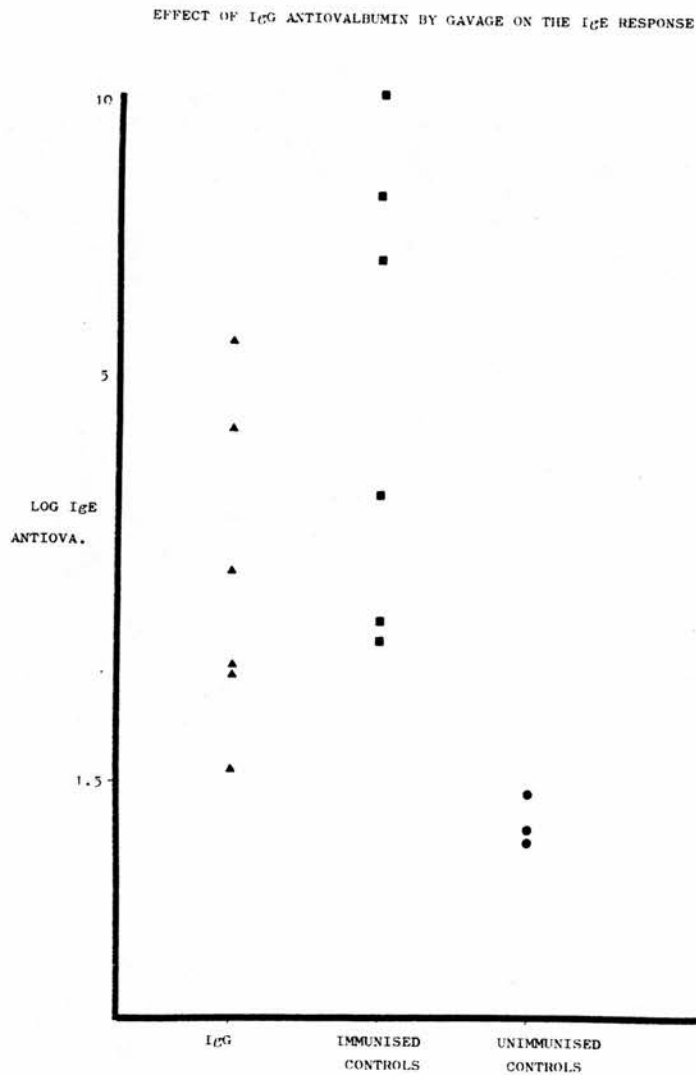
Since the number of animals was small, an additional parametric test of significance was performed after log transformation of the data. This proved to be significant ( $t = 2.75$  p < 0.05.).

### Discussion

Suppression of the IgE antiovalbumin response has been shown in rats suckled by ovalbumin-sensitized females, confirming the results of Jarrett and Hall (1979). A comparable degree of suppression also occurred in rats fed serum from immunized animals suggesting that antiovalbumin antibody ingested during the suckling period prevents a subsequent IgE antiovalbumin response. Rats of weaning age have IgG<sub>2A</sub> levels in serum

LEGEND TO FIGURE 19:

Secondary IgE antiovalbumin response is shown in rats treated before immunization with IgG antiovalbumin by gavage and in immunized and unimmunized litter mate controls. Suppression of IgE response in the IgG treated group is significant (log transformed data  $t = 2.75$   $p < 0.05$ ).



approaching those in adult rats (mean 6.32 mgs/ml) (McGhee et al.1975) yet only a small amount of this immunoglobulin is acquired *in utero* and most is absorbed from milk ingested during suckling. Rat pups probably absorb IgG from ingested serum as well because they have Fc receptors for IgG on intestinal mucosa (Borthistle et al. 1977). In an experiment limited in numbers by the small amount of antibody preparation available, the suppressive effect of IgG from immune serum was arguably significant. The dose of IgG was small, only 1.3% of the calculated amount of IgG normally acquired from milk, and greater suppression may have occurred with a larger dose of antiovalbumin antibody. It is probable that IgG antiovalbumin antibody suppresses the IgE antiovalbumin response though it is not known if there are other suppressive factors operating. Other mechanisms of suppression may be involved since Jarrett and Hall (1979) demonstrated that circulating maternal antiovalbumin antibody was only just detectable in pre-immunized offspring. In addition they found more IgG antiovalbumin and total antibody in immunized rats from immunized mothers, suggesting that suppression was confined to the IgE response.

Cross fostering experiments showed that suppression of IgE response in litters suckled by immunized lactating females was less complete than in non-crossed litters. This may have resulted from inter-litter variation in responsiveness but it is possible that small amounts of transplacentally-acquired immunoglobulin may have contributed to suppression. That the human foetus acquires immunoglobulin by this route (Brambell, 1966) suggests that prevention of sensitization could occur in the neonate; this would be expected to be antigen specific and dependent on the titre of circulating specific antibody of maternal origin in neonatal blood. It would also be unrelated to infant feeding practise since the human infant does not absorb milk immunoglobulin.

## Chapter 6

### ANTIGEN SPECIFIC AND ANTIGEN NON-SPECIFIC INFLUENCE OF SUPPLEMENTARY INFANT FEEDS ON THE IgE AND IgG ANTIBODY RESPONSES

#### INTRODUCTION

Jarrett and Hall (1979) showed by cross fostering experiments that the milk of ovalbumin-sensitized female rats suppressed the IgE antiovalbumin response in suckled offspring, and this has been confirmed (Chapter 5). However, the relevance of this to human atopic allergy is uncertain because the protective effects of human milk seem to be antigen non-specific. Foods other than cows milk, for example eggs, cause eczema (Atherton et al. 1978) and cows milk does not usually provoke asthma which may also be prevented by exclusive breast feeding (Chandra, 1979). In addition, there is no evidence at present that protection is confined to the milk of sensitized mothers. The effect of supplementary feeds of a cows milk preparation on the IgE and IgG response to ovalbumin was therefore investigated.

Neonatal antigen contact provokes antigen specific allergy in susceptible human infants (Bjorksten, Suoniemi and Koski, 1980), and cows milk feeds at birth appear to provoke sensitization to several environmental antigens (Chandra, 1979). Such effects persist throughout much of childhood, since IgE antibody responses are usually maintained by repeated exposure to the sensitizing antigen (Orren, Dowdle, 1975). In contrast, repeated administration of small doses of sensitizing antigen to rats suppress the IgE response and stimulate the IgG one (Jarrett, Stewart, 1974). In addition, the secondary IgE response is of short duration. Such effects have not been investigated in rats fed supplementary feeds of cows milk preparations so their possible effects on rat IgE responses in later life were studied. That the IgE responses were harmful to the rat was shown by production of anaphylactic symptoms with antigen

challenge. Atopic symptoms are more common in bottle-fed infants, and this clinical observation was extended to the supplementary fed rat. The young rat is protected against sensitization by milk from immunized mothers though the mechanism of protection is probably different from that in breast-fed infants. The possible effects of supplementary feeds of cows milk-based preparations in antagonising the suppressive effects of milk from immunized females has not previously been studied and is reported here.

The most commonly encountered food allergy in human infants is that to cows milk protein antigens. Despite frequent exposure to these proteins, a prospective study demonstrated the prevalence of symptoms to be less than two percent, and unlike most atopic allergies in later life, sixty percent of symptomatic children had spontaneous resolution of symptoms before two years of age (Jakobssen, Lindberg, 1979). Cows milk protein allergy is unusual in other respects; reaginic antibody probably accounts for a minority of hypersensitivity symptoms and only a minority of patients have positive prick tests to cows milk protein (Hill, Davidson, Cameron, Barnes, 1979). Though it is possible for mucosal reaginic hypersensitivity to exist without detectable systemic or cutaneous IgE antibody (Huggins, Brostoff, 1975) it is more likely that other hypersensitivity mechanisms, such as circulatory immune complexes, mediate some of the symptoms. The possible production of IgE antibody to one of these proteins,  $\beta$  lactoglobulin, in rats supplemented with a cows milk-based preparation was investigated.

EFFECT OF SUPPLEMENTARY FEEDS ON THE IgE AND IgG  
ANTIOVALBUMIN RESPONSE

Litters from six pregnant HL rats (Animal Suppliers Ltd.) were divided into supplement-fed and control groups. Supplements of the cows milk-based preparation were prepared as described previously (Chapter 3, page 38) and were fed to rat pups by stomach tube in doses of 0.1 ml/G Body weight, equivalent to half their daily nutritional requirement (Table 2, page 39). Supplements were given during a twelve-hour daily period between the third day of life, to weaning at twenty-one days. Sixteen out of forty-two supplement fed rats died of complications of the gavage, most within the first week of life. Control animals suckled without interruption and were not gavaged. After weaning all the rats received Oxoid breeder rat diet and water and libitum. Neither the supplementary feed nor the breeder diet contained ovalbumin (Chapter 2, page 34).

Ten supplement-fed rats and eleven controls were immunized with 1.0 µg ovalbumin and  $10^{10}$  Bordetella pertussis by intraperitoneal injection on day 22. Another litter-matched pair of supplemented and control groups were similarly immunized with 10 µgs ovalbumin. A second intraperitoneal injection of 1.0 µg ovalbumin was given on day 56 and blood taken by cardiac puncture four days later. The secondary IgE antiovalbumin response was determined by PRAST. Agglutinating antiovalbumin in the sera, with and without pre-treatment with Dithiothreitol (Chapter 2, page 33) was assayed with ovalbumin-coated, chromic chloride-treated erythrocytes. This treatment reduced the titre by  $> 1$  in only two samples. An identical experiment was performed with thirty-two supplement-fed and thirty-five control HL rats from another supplier (OLAC Ltd.) (Experiment 2), Table 4).

IgE antiovalbumin values (counts bound/counts bound by newborn serum) and DTT-resistant agglutinating antibody ( $-\log_2$  titre) are shown in Table 4 (Experiment 1) and Figures 20 and 21. Four of the eleven control unsupplemented animals (Animal Suppliers Ltd.), immunized with 1.0  $\mu$ g ovalbumin, had IgE antiovalbumin values less than the highest value in unimmunized rats (1.4). All ten supplement fed animals had values above this and the median value was significantly higher than that of the unsupplemented controls ( $p < 0.01$ ). (Mann Whitney U .) This finding was confirmed in the second experiment with HL rats from a different colony (OLAC) (Table 4;  $p < 0.05$ ).

Fewer of the control rats had DTT-resistant agglutinating antibody (presumably IgG antiovalbumin) than did the supplement-fed animals (Table 4 and Figure 21;  $p = 0.002$ , Fisher exact test).

In sixteen supplement-fed rats and fourteen controls, injected with 10  $\mu$ g ovalbumin, there was no significant difference in the IgE response. The agglutinating antibody titre was significantly lower in the supplement-fed rats ( $p < 0.01$ , Mann Whitney U .). There was no significant correlation between IgE and IgG values in any of the groups ( $r$  ranged from 0.15 to 0.22).

LEGEND TO TABLE 4:

Median IgE (counts bound/counts bound by newborn serum) and DTT resistant agglutinating antibody ( $-\log_2$  titre) in supplemented (S) and control (C) rats immunized with 1.0 or 10  $\mu$ g. ovalbumin from the separate experiments (1 and 2). Median IgE antiovalbumin values in eighteen unimmunized adult rats was  $1.1 \pm 0.3$ . (Mann Whitney U and Fisher exact tests.)



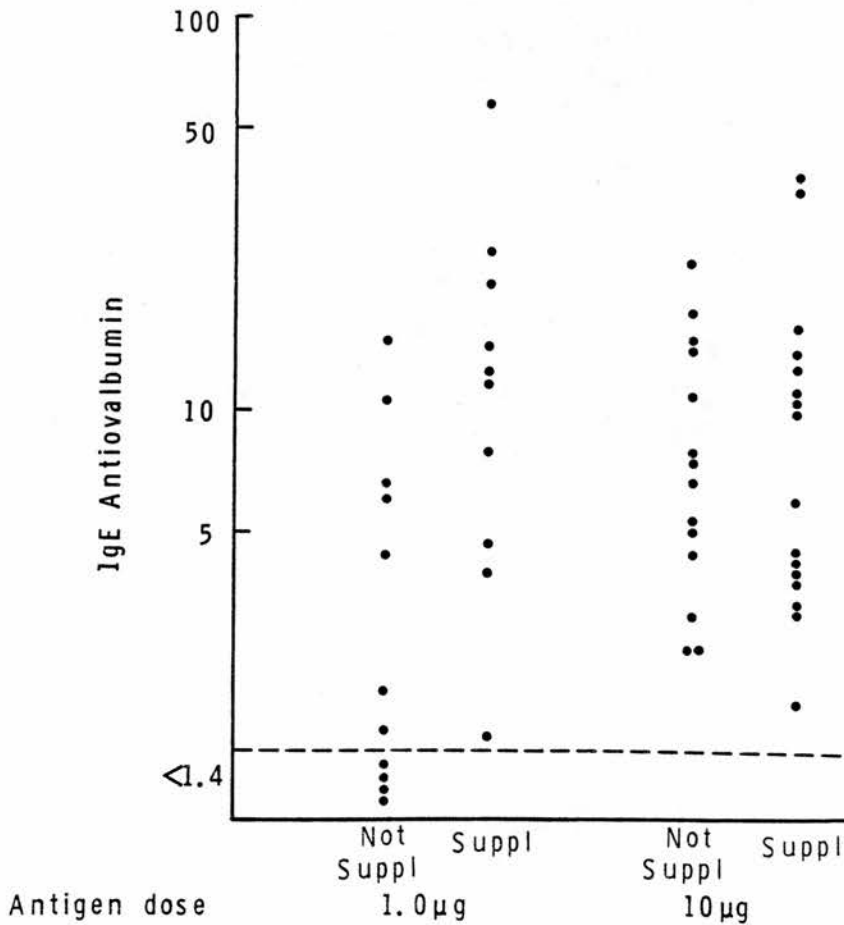
TABLE 4:

Experiment 1										Experiment 2					
IgE		Antioval- bumin		IgG		Antioval- bumin		IgE		Antioval- bumin					
OVALBUMIN DOSE	1.0μg	10.0μg		1.0μg		10.0μg		1.0μg		10.0μg					
FEEDING CATEGORY	S	C	S	C	S	C	S	C	S	C	S	C			
NO. OF RATS	10	11	16	14	10	11	16	14	15	13	17	22			
MEDIAN	12.0	2.1	7.8	6.9	11	<3	10	16	8.8	2.6	5.2	5.8			
P	<0.01	NS		*0.002		<0.01		<0.05		NS					

LEGEND TO FIGURE 20:

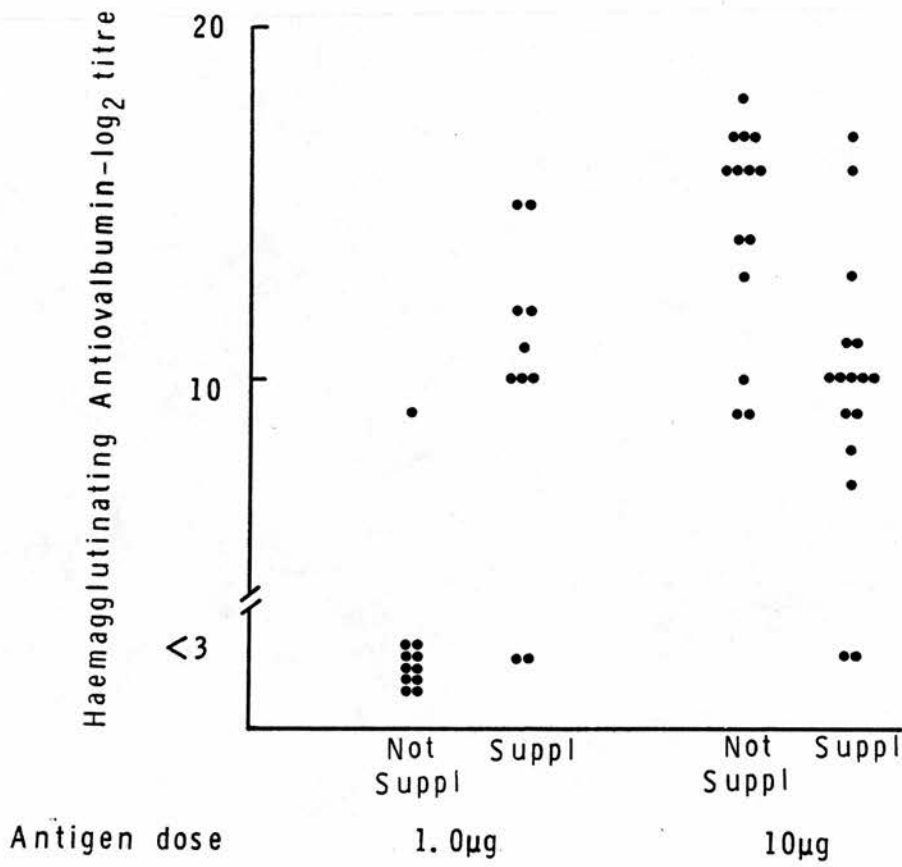
IgE antiovalbumin antibody (counts bound by sample/  
counts bound by newborn rat serum) in sixty day old rats,  
following immunization with ovalbumin in doses of  $1.0 \mu\text{g}$   
or  $10 \mu\text{g}$  with adjuvant (Experiment 1).

Rats which had received cows milk-based supplementary feeds  
and litter mates who did not are shown. The highest value  
observed in unimmunized rats (1.4) is indicated.



LEGEND TO FIGURE 21:

DTT-resistant haemagglutinating antiovalbumin antibody  
( $-\log_2$  titre) in supplemented and control rats immunized  
as described in Figure 20 (Experiment 1).



Effect of the Supplementary Feeds on Immunization with Ovalbumin Repeated later in Life.

The preceding experiment demonstrated that the secondary anti-ovalbumin responses are abnormal in supplement-fed rats. The response to subsequent antigen exposure later in life was explored in two different experiments. Five supplement fed and five unsupplemented controls were immunized with 10  $\mu$ g ovalbumin as described previously. The secondary IgE response was determined. IgE antiovalbumin antibody was present in all the rats at sixty days of age (mean 20.8 counts bound/counts bound by newborn serum. Range 4-54). There was no significant difference in the mean values of the two groups. At ten weeks of age they then received a third intraperitoneal injection of 1.0  $\mu$ g. ovalbumin; 10 ml. blood was withdrawn by cardiac puncture fourteen days later. A fourth injection of 1.0  $\mu$ g ovalbumin was given immediately and blood withdrawn four days later. Adjuvant was not given with the third or fourth injections. IgE values, assayed in the same batch, are shown in Table 5. The IgE antiovalbumin values at twelve weeks of age and after the third ovalbumin dose were much lower than those of the secondary response. No additional IgE antibody was detected four days after the fourth injection of ovalbumin in either the rats who had received supplements or controls.

LEGEND TO FIGURE 22:

Immunization scheme to investigate the response of rats to a second immunization with ovalbumin and pertussis as described in the method (page 75).

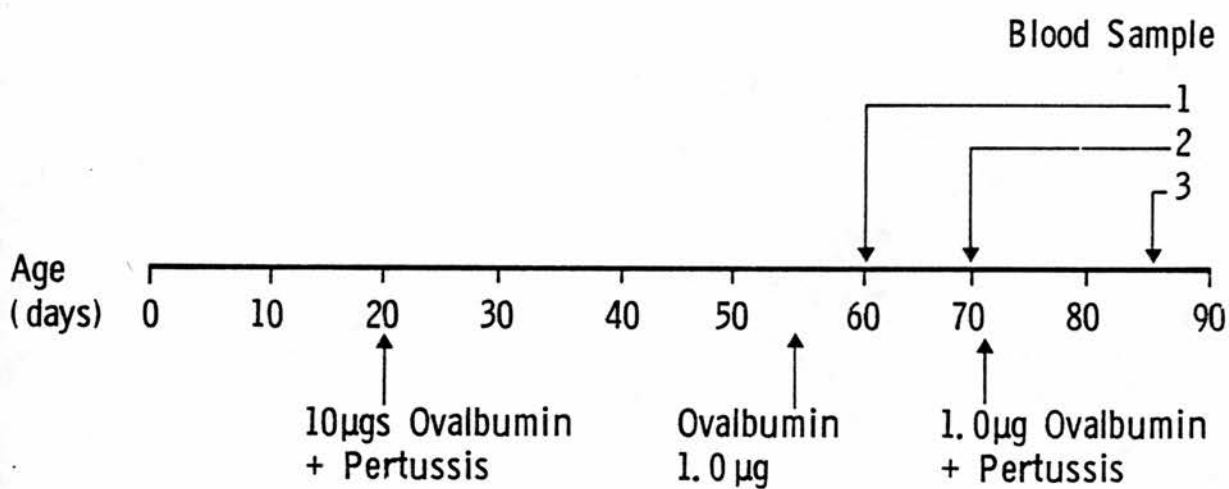


TABLE 5:

MEAN AND RANGE OF IgE ANTIOVALBUMIN VALUES AFTER 2,3 AND 4 INJECTIONS OF OVALBUMIN, IN SUPPLEMENT-FED AND CONTROL RATS

	Secondary Response	Tertiary Response	Quaternary Response
<u>n</u>			
Supplemented 5	20.7	1.52 (0.7 - 2.1)	1.72 (0.7 - 2.2)
Controls 5	20.9	1.47 (0.7 - 1.8)	1.5 (0.75 - 1.9)

p(S-C)NS

The observations in unsupplemented rats are similar to the report by Jarrett and Stewart (1974) that IgE antiovalbumin values decline rapidly after the secondary response and cannot be boosted by further injections of antigens. My data shows that supplement-fed rats behave similarly.

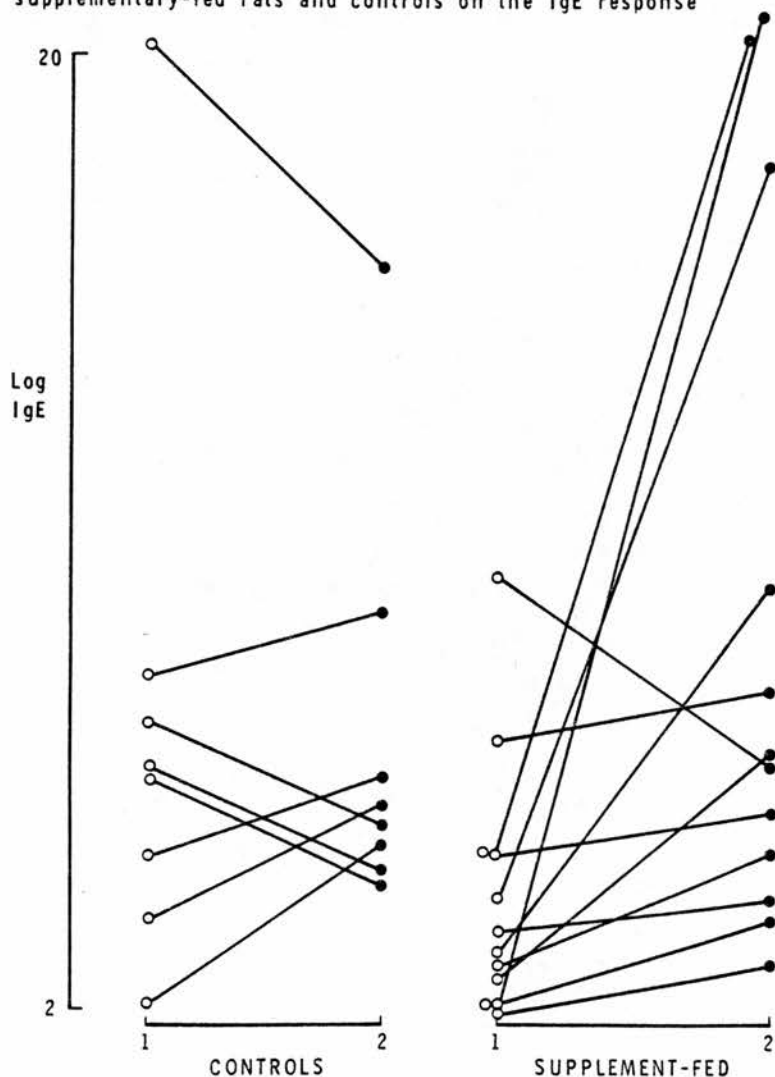
The response of a group of twelve supplement-fed rats and eight controls to later immunization with ovalbumin and adjuvant was studied (Figure 22). All were immunized with 10 µgs. ovalbumin with pertussis at weaning, and IgE antiovalbumin was detected at eight weeks of age (Mean 9.3 range 4.6 - 31.8). Blood was withdrawn at ten weeks and the animals were then immunized for a second time with intraperitoneal ovalbumin (1.0 mgs) and  $10^{10}$  Bordetella pertussis. There was a significant further increase of IgE in the supplemented group ( $t = 3.77$   $p < 0.005$ ) but not in the unsupplemented controls (Figure 23).

The low IgE antiovalbumin values observed in both groups before the second immunization did not differ significantly in the supplemented and control rats (Manns Whitney  $U = 25$ ,  $p$  NS) so the different response was not the result of differences in the values before immunization.

LEGEND TO FIGURE 23.

The IgE response to a second immunization with ovalbumin and adjuvant in supplement-fed rats and unsupplemented controls is shown. The response to immunization is significant in the supplemented group (paired  $t$  test  $=3.77$   $p < 0.005$ ).

Effect of a second immunisation with Ovalbumin in Pertussis in supplementary-fed rats and controls on the IgE response



#### ANAPHYLAXIS IN SENSITIZED RATS

Rat mast cells, with IgE antibody on their surface, release histamine and other inflammatory mediators on contact with antigen (Karlsson, 1978). Local and systemic hypersensitivity reactions are produced (Ovary, 1952) (Phillips-Quagliata, 1972). However, it is possible that other classes of immunoglobulin also mediate anaphylaxis. In the human both IgE and IgG4 antibody produce symptoms (Gwynn et al. 1979). Since human infants, receiving cows milk preparations, have more atopic symptoms than do breast fed ones (Mathew et al. 1977), the possible role of feeding supplements of cows milk preparation to young rats in increasing hypersensitivity symptoms was investigated.

Ovalbumin sensitized, supplement-fed and unsupplemented rats were challenged by intravenous injection with ovalbumin. Of thirteen newborn HL rat pups, seven received cows milk-based supplements and the rest were suckled normally. At weaning, 1.0 µg. ovalbumin and  $10^{10}$  Bordetella pertussis were injected intraperitoneally. Median secondary IgE anti-ovalbumin values at sixty days of age were 3.35 (range 1.3 to 13.6 counts bound/counts bound by newborn serum) in supplemented rats and 2.52 (range 1.4 to 6.0) in unsupplemented rats. Median haemagglutinating antiovalbumin titres in the same sera were 16 (range 15-18- $\log_2$  titre) and 15 (range 12-18) respectively. Control groups consisted of four rats with haemagglutinating antiovalbumin (Median 17.5 -  $\log_2$  titre) but no detectable IgE antiovalbumin antibody, and six unimmunized rats. At day 64, the animals were restrained in a cage and injected via the tail vein with 10 mgs. ovalbumin (Sigma 5 x crystallized) in 1 ml. of 0.15N saline. Reactions were classified by an independent observer (B.J.Gregory) into four categories:



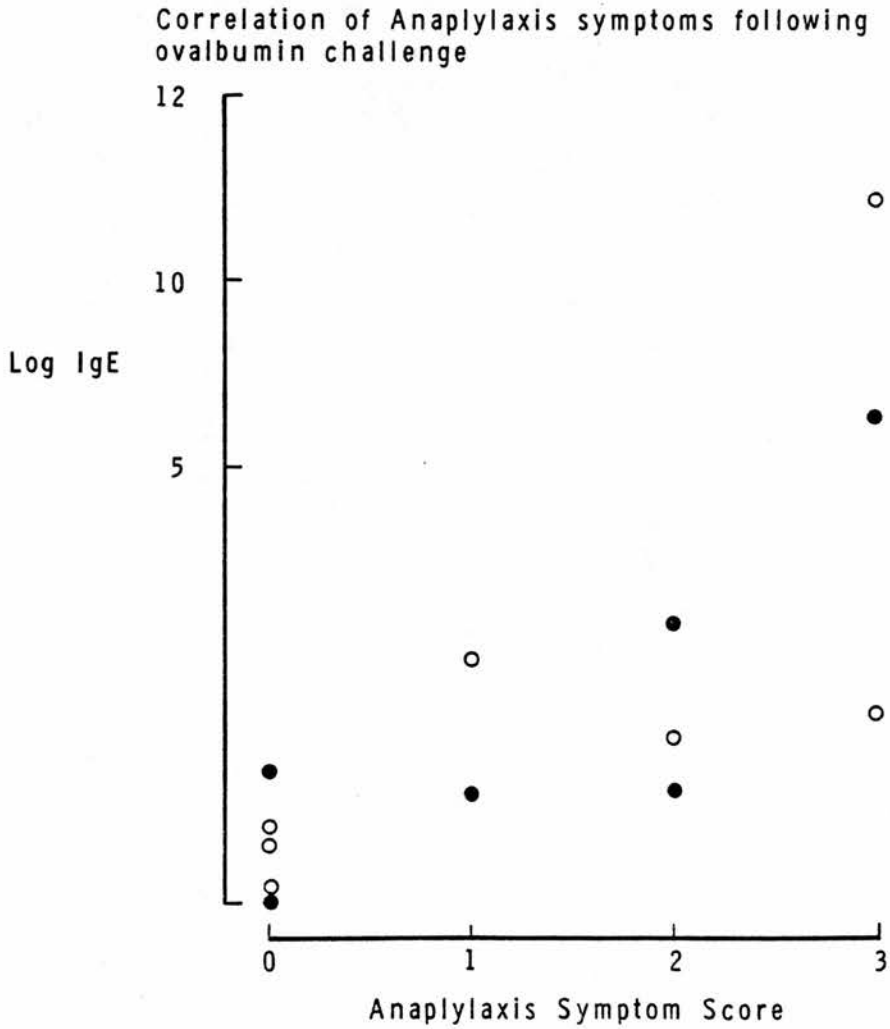
- |         |   |  |
|---------|---|--|
| Score 0 | - | no effect  |
| 1       | - | mild transient tachypnoea and lethargy of immediate onset and less than thirty minutes duration. |
| 2       | - | severe immediate symptoms of tachypnoea, peripheral cyanosis and loss of consciousness.          |
| 3       | - | death within sixty minutes of injection.   |

There was a significant correlation between IgE antibody value and severity of symptoms (Figure 24.) (Spearman's rank correlation coefficient  $r = +0.825$   $p < 0.005$ ). Four of the seven supplement-fed rats had IgE antiovalbumin antibody values greater than values in unimmunized rats ( $>1.4$  counts bound/counts bound by newborn serum) and these four had anaphylaxis symptoms, as did four of the five unsupplemented rats who had IgE antibody (Figure 24). There were no significant differences in antibody values, symptom scores or fatalities in the supplemented and unsupplemented rats (Chi squared contingency table analysis = 0.2146  $p = 0.9$ ). Ovalbumin challenge in six unimmunized rats had no observed effect. Mild symptoms (score 1) were seen in one of four animals who had haemagglutinating ovalbumin but no IgE antiovalbumin antibody.

Though the correlation of symptoms with IgE antibody was highly significant, the challenged animals were selected to have a range of antibody values. In this experiment, symptoms were not shown to be influenced by previous exposure to cows milk-based supplements, but the result is open to question because the animals were not randomly chosen and the number of challenged rats who had IgE antibody was small. The symptomatic effect of haemagglutinating antibody was minimal.

LEGEND TO FIGURE 24:

Anaphylaxis symptoms in supplemented (O) and un-supplemented (●) rats with IgE antiovalbumin antibody intravenously challenged with 10 mgs. ovalbumin. The correlation of symptoms with IgE antiovalbumin values is significant (Spearman's  $r +0.825$   $p < 0.005$ ).

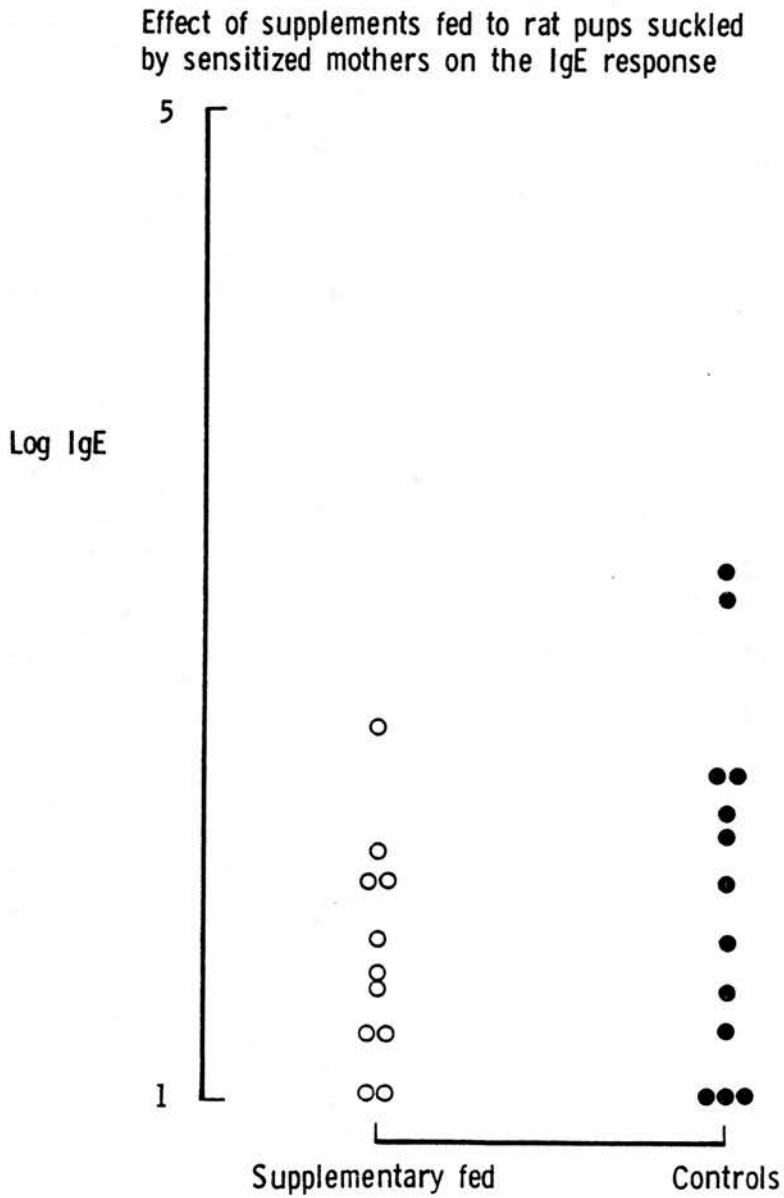


SUPPRESSION OF IgE RESPONSE IN SUPPLEMENTARY FED RATS  
SUCKLED BY SENSITIZED FEMALES.

The suppression of IgE antiovalbumin response in rats suckled by ovalbumin-sensitized females (Jarrett, Hall, 1979), probably results from the passive transfer of antiovalbumin antibody (Chapter 5, Figure 19). Suckling from immunized females for only seven days produced the effect (Jarrett, Hall, 1979). The supplementary feeding regimen described in Chapter 3 which reduces by half the expected amount of maternal antibody acquired by the rat pup, would therefore presumably be insufficient to influence this suppression of IgE response. However abrogation of suppression may occur if supplementary feeds increase IgE responsiveness as in rat pups suckled by non-sensitized females (Chapter 6, Figures 20 & 21). This possibility was investigated in litters from two HL females who had been immunized with 10 mgs. ovalbumin in pertussis adjuvant. One group of eleven pups from both the litters received supplementary feeds of the cows milk preparation as described previously. Thirteen controls suckled normally from the sensitized females until weaning. All the animals were immunized with 1.0  $\mu$ g. ovalbumin and  $10^{10}$  Bordetella pertussis by intraperitoneal injection at twenty-one days. The secondary IgE responses at sixty days were similar for the two groups (Figure 25) so the suppression of IgE response in rats suckled by sensitized females was not antagonised by supplementary feeding.

LEGEND TO FIGURE 25:

Secondary IgE antibody response in supplement-fed rats and unsupplemented controls intraperitoneally injected with 1.0  $\mu$ g. ovalbumin and pertussis adjuvant at weaning. Both groups were suckled by ovalbumin-sensitized females.



ANTI  $\beta$  LACTOGLOBULIN ANTIBODY IN SUPPLEMENTARY-FED RATS

Cows milk contains approximately 2.5g/litre  $\beta$  lactoglobulin (Lynster, 1972). The supplementary feed of a cows milk-based preparation, described in Chapter 3, was tested for the presence of  $\beta$  lactoglobulin. Doubling dilutions of the supplementary feed were placed in wells in an agar plate and allowed to diffuse for twenty-four hours with solution of  $\beta$  lactoglobulin (1mg/ml) and neat rabbit anti-bovine  $\beta$  lactoglobulin (kindly donated by Dr.M.W.Turner). Immuno-diffusion showed a reaction of identity between the supplementary feed and the  $\beta$  lactoglobulin.

Twenty-four suckled HL rat pups received supplementary feeds of the cows milk-based preparation between day three and weaning. They were immunized by intraperitoneal injection with 1.0  $\mu$ g ovalbumin and pertussis adjuvant on day 22. All had a secondary IgE antiovalbumin response. The same sera were stored at  $-70^{\circ}\text{C}$  and tested for anti  $\beta$  lactoglobulin antibody. Six unsupplemented pups were immunized on day 22 with 1.0  $\mu$ g.  $\beta$  lactoglobulin and  $10^{10}$  Bordatella pertussis by intraperitoneal injection. Blood was withdrawn four days after a second injection of 1.0  $\mu$ g.  $\beta$  lactoglobulin at fifty-six days of age. Six adult HL rats were immunized with three subcutaneous injections of 1.0 mg.  $\beta$  lactoglobulin in Freund's complete adjuvant. Sera from all the animals were tested for IgE anti  $\beta$  lactoglobulin antibody by PRIST using cyanogen bromide-activated  $\beta$  lactoglobulin-coated paper discs (see page 30). IgE anti  $\beta$  lactoglobulin was detected in five of six rats immunized with  $\beta$  lactoglobulin and pertussis (Median 4.1, range 2.15 - 11.6. counts bound/counts bound by newborn serum). None of the twenty-four supplemented rats had a significant IgE response (range 0.6 - 1.24 counts bound/counts bound by newborn serum), four of these sera were negative in passive cutaneous anaphylaxis tests.

Haemagglutinating anti $\beta$ lactoglobulin antibody was detected by chromic chloride treated  $\beta$  lactoglobulin-coated human erythrocytes (Kofler, Wick, 1977). Anti $\alpha$ lactoglobulin also agglutinated these cells, anticasein serum did not (Table 6). (Antisera kindly donated by Dr.M.W. Turner.)

TABLE 6:

SPECIFICITY OF THE HAEMAGGLUTINATING ANTI  
 $\beta$  LACTOGLOBULIN ASSAY

<u>Antiserum</u>	<u>Haemagglutinating titre</u> ( $-\log_2$ )
Human anti $\beta$ lactoglobulin	$>8$
Human anti $\alpha$ lactoglobulin	$>8$
Human anticasein	$<3$
Human antiovalbumin	$<3$
Normal human serum	$<3$
Rat anti $\beta$ lactoglobulin )	$>8$
Normal rat serum ) absorbed with	
	50% human
	RBCs
	$<3$

Sera from the twenty-four supplementary-fed rats were absorbed with 50% erythrocytes in phosphate buffered saline. Doubling dilutions were mixed with equal volumes of the  $\beta$ lactoglobulin-coated erythrocytes in wells in microtitre plates, together with a control for non-specific agglutination (Chapter 2, page 33). The haemagglutinating anti  $\beta$  lactoglobulin titre was  $<3$  in all the samples tested. Six animals immunized with  $\beta$ lactoglobulin in freunds complete adjuvant had haemagglutinating titres  $>8$ . Neither IgE nor haemagglutinating anti  $\beta$  lactoglobulin antibody was detected in any of the supplementary fed, ovalbumin sensitized rats though the feed contained  $\beta$  lactoglobulin. Conventional immunization procedures with injected antigen and adjuvant did produce an antibody response

## Discussion

The experiments described in this chapter have shown that supplementary feeds to suckling rats have several immunological effects. The most important, confirmed in two separate experiments, is that of an antigen non-specific increase of the IgE and IgG antibody responses. Young HL rats readily produce an IgE response to injected antigen with adjuvant and the response is dependent on antigen dose (Jarrett, Stewart, 1974). Supplementary feeds appear to lower the threshold of sensitization so that suboptimal antigen doses with adjuvant provoke IgE antibody responses. This observation is similar to the concept of allergic breakthrough described by Katz (1979) though the mechanism is different because I studied a high IgE responding animal strain and did not manipulate lymphocyte responses by pretreatment or irradiation. The effect of supplementary feeds increasing IgE response is similar to clinical observations of such feeding in human infants (Chandra, 1979) (Saarinen et al. 1979).<sup>(a)</sup> Furthermore, the increase in both IgE and IgG antibody is comparable with allergic antibody responses in human atopy (Platts-Mills et al. 1978). Unlike human atopic allergy, IgE responses in rats require injected adjuvant and the antibody response to antigen without adjuvant was insignificant. That supplementary feeds of cows milk-based preparations influence the response to injected antigen suggests that such feeding has effects which are not confined to antigen handling at mucosal surfaces.

Supplementary-fed rats produced less IgG antiovalbumin than unsupplemented controls when immunized with a larger dose of ovalbumin (10 µgs), but the increase of IgE response was similar in both groups. This curious finding of an antigen non-specific decrease of IgG production has been shown following skin contact with antigen (Thomas, Watkins, Asherson, 1978). Its mechanism and possible significance

for subsequent antibody response is uncertain. Rats injected with repeated small doses of antigen usually suppress IgE responses and produce IgG antibody (Jarrett and Stewart, 1974). This finding was confirmed in normally reared rats where a third injection of ovalbumin with or without pertussis adjuvant failed to provoke a tertiary IgE response. Supplement-fed rats showed significant differences in the tertiary response to antigen with adjuvant suggesting that supplementary feeds influence IgE responses later in life. This prolonged influence of supplementary feeds on IgE response has some resemblance to the clinical observations that in atopic human infants primed by neonatal antigen exposure, subsequent antigen contact increases the IgE response (Bjorksten et al. 1980).

The pathological significance of rat IgE antibody is open to question; one function of this immunoglobulin class is that of protection against parasitic infection (Jarrett, Stewart, 1972). Support for the role of IgE antibody in hypersensitivity reactions was provided by the demonstration of anaphylaxis symptoms in sensitized rats. It was not possible to demonstrate conclusively that IgE antibody was responsible for anaphylaxis because all the symptomatic animals had more than one class of antiovalbumin antibody but it is evident that, as in humans, sensitization to specific antigen has harmful consequences. (Wide, Bennich, Johanson, 1967). That the hypersensitivity symptoms were of a similar degree in supplement fed and unsupplemented rats was surprising; human infants fed with cows milk preparations have more atopic symptoms than breast fed ones (Mathew et al. 1977). The hypersensitivity symptoms in human infants may not all be IgE mediated; other classes of antibody (Gwynn et al. 1979) and circulating immune complexes probably contribute to atopic disease (Brostoff et al. 1979). In addition, symptoms were studied in rats chosen for IgE antibody levels and differences in



supplementary fed and unsupplemented immunized rats may have been shown by a larger study.

The relevance of suppression of specific IgE response by sensitized lactating rats to human atopic allergy is uncertain because the protective effects of human breast milk appear to be antigen non-specific (Mathew et al. 1977) (Saarinen, Kajosaari, Bachman, Siimes, 1979)<sup>(a)</sup>. There is no evidence that this protection is confined to the milk of sensitized mothers and human infants do not absorb milk immunoglobulin from the gut (Ammann, Stehm, 1977). Supplementary feeds have an antigen non-specific effect on the antigen specific IgE response which has some resemblance to the clinical observations in human infants. Such feeding does not influence the antigen specific suppression of IgE response by maternal milk whereas even small amounts of cows milk fed to susceptible human infants predisposes to atopic allergy (Saarinen, Kajosaari, Bachman, Siimes, 1979)<sup>(a)</sup>.

One criticism of the supplementary feeding experiments was that the control animals were not gavaged. It is possible that the increased IgE response resulted from gastrointestinal infection or inhalation of milk into the lungs. The lack of such an effect in the experiment using a higher dose of injected antigen suggests that aspects of the manipulation other than the milk supplement were not responsible for differences in IgE responses. The ideal control, gavaging with rat milk, was not possible. The considerable mortality of the gavaged rats is unlikely to explain the differences; if the deaths were anaphylactic they would select vulnerable animals and reduce the observed effect. It is more likely, however, that deaths resulted from physical injury because most occurred in the first week of life before sensitization is likely to have developed.

There is some suggestion that the immature intestine of presumably non-sensitized human babies can absorb greater quantities of ingested food protein than older infants or adults (Grusky, Cooke, 1955)(Walker,1978). The consequences of such antigen contact early in life are unpredictable but they are sufficient to immunize since antibodies to food proteins have been demonstrated in low titres in most healthy people (Peterson, Good, 1965). The presence of secretory IgA antibodies to cows milk proteins in breast milk suggests that antigen absorption may be modified in those breast fed infants receiving supplements of cows milk (Hanson et al. 1977). I did not investigate the possibility that rat milk partially protects the supplementary fed rat pup against absorption of cows milk proteins because a suitable control group, those receiving an entirely cows milk-based diet, was not available.

In animals, oral immunization may evoke a local immune response involving secretory IgA antibody as well as a state of systemic hyporesponsiveness or 'tolerance' in which the animals are incapable of mounting an appropriate serum antibody response when the same antigen is administered parenterally (Thomas, Parrott, 1974)(Swarbrick, Stokes, Soothill, 1979). Food allergy may be regarded as a failure of this orally induced tolerance. Allergy to cows milk did not occur in supplementary fed rats since they did not produce IgE and haemagglutinating antibody to cows milk protein, and their sera did not produce cutaneous hypersensitivity reactions. Secretory antibodies to cows milk proteins were not studied in the supplemented animals so it is not known if the lack of systemic antibody response was the result of tolerance or a failure to immunize appropriately with adjuvant.

## Chapter 7

### THE MECHANISM OF THE EFFECT OF SUPPLEMENTARY FEEDS ON IgE RESPONSE

#### INTRODUCTION

Experiments in the preceding chapter showed an antigen non-specific effect of cows milk-based feeds on IgE and IgG antiovalbumin responses. A possible mechanism for the effect may be an adjuvant one. In human infants, supplementary feeds of cows milk preparations change faecal flora, producing an overgrowth of gram-negative bacilli (Bullen, Tearle, Steward, 1977). Consequent release and absorption of endotoxin might have adjuvant effects on antibody responses to many antigens to which the infant is exposed. It is implicit that responses, not only to food antigen, but to that absorbed from other mucosal surfaces, will be influenced. There is no data to show that supplements of cows milk-based preparation fed to rat pups influence intestinal bacterial colonization, but their possible adjuvant effects on IgE responses to injected ovalbumin were explored. Supplementary feeds may have other effects such as antigen absorption from the gut. There is some evidence that, in unimmunized animals, competition for antigen uptake occurs (Roberts, Reinhardt, Paganelli, Levinsky, 1980). I therefore investigated the effect of the supplementary feeds on immunization with oral ovalbumin.

Bottle-fed human infants have higher IgE levels than breast-fed ones (Saarinen et al. 1979)<sup>(b)</sup>, suggesting that cows milk feeds have antigen non-specific effects, increasing total IgE synthesis. This has not been confirmed by other studies in infants of atopic parents (Soothill et al. 1976), and in infants with no family history of allergy (Juto, Bjorksten, 1980). The possible association between total IgE and IgE antibody was explored in supplement fed and unsupplemented rats.

ADJUVANT EFFECT OF ENDOTOXIN AND OF THE SUPPLEMENTARY FEED

One possible mechanism of the supplementary feeds increasing the IgE response may be to encourage the intestinal growth of endotoxin producing bacteria (Bullen, Tearle, Steward, 1977) and consequent absorption of endotoxin, which has adjuvant activity in many species (Morrison, Ryan, 1979). The adjuvant effect of endotoxin, both injected and ingested, on IgE responses in rats was investigated. In preliminary experiments to determine the optimum dose of endotoxin, fifteen weaning HL rats received intraperitoneal E.Coli Lipopolysaccharide (LPS) (Sigma Chemicals) in 0.15N saline in doses of 1,5,10,50 or 100 µgs together with 10 µgs ovalbumin. The secondary IgE responses are shown in Table 7. The highest non-lethal dose of LPS adjuvant for optimal IgE responses was 5 µgs, and this dose was used in subsequent experiments. LPS was tolerated orally in doses as large as 1000 µgs in weaning rats without symptoms (twenty-one days). A dose of 100 µgs. was chosen for subsequent oral immunization experiments; this dose is comparable with previously described immunizing regimens (Farthing, Holt, 1962).

TABLE 7:

IG E ANTIOVALBUMIN RESPONSE TO DIFFERENT DOSES OF LPS  
ADJUVANT INJECTED WITH 10 µgs. OVALBUMIN

n.	LPS Dose(µgs)	Mean IgE Antiovalbumin	Range
3	1	3.2	2.69 - 3.7
3	5	6.2	4.36 - 8.9
3	10	3.1	2.3 - 3.86
3	50	4.4	4.26 - 4.87
3	100	24.8 (fatal in 2/3)	

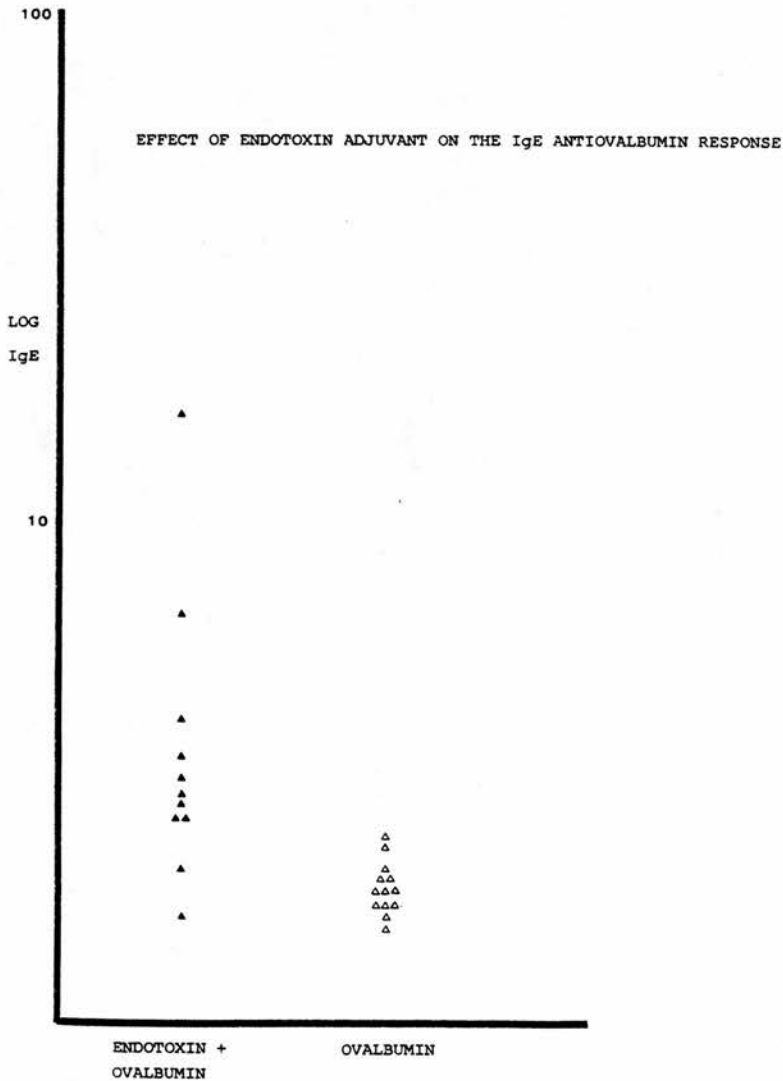
(Mann, Whitney U (1 and 5 µgs) and (5 and 10 µgs)  $p = < 0.05$

That the animals injected with the highest dose of LPS (100 µgs) rapidly died suggests that they suffered endotoxin shock.

LEGEND TO FIGURE 26:

The IgE response is shown in young rats immunized by intraperitoneal injection of ovalbumin with LPS endotoxin and in controls injected with the same dose of ovalbumin without LPS. The response in the two groups is significantly different.

(Mann Whitney  $U = 14$   $p < 0.01$ .)



#### Adjuvant effect of Endotoxin

Three litters of HL rats were divided into three groups at weaning (twenty-one days), which received 10  $\mu$ gs intraperitoneal ovalbumin on day 22 alone or with 5  $\mu$ gs LPS by intraperitoneal injection, or with 100  $\mu$ gs LPS by gavage. All animals were injected intraperitoneally with 1.0  $\mu$ g ovalbumin without adjuvant on day 56 and tested four days later for IgE antiovalbumin response. The secondary response to injected antigen was significantly greater in the LPS injected group (Median 2.9 range 1.6 - 10) than in controls who did not receive adjuvant (Median 1.8 range 1.5 - 2.3 counts bound/counts bound by newborn serum) (Fig. 26 Mann Whitney  $U = 14$   $p < 0.01$ ). The response in nine rats treated with oral adjuvant and injected antigen did not differ from the controls; these results are not shown in the figure. Median value was 1.6 counts bound/counts bound by newborn serum, range 1.5 - 1.8.

Though adjuvance of IgE response by LPS has been demonstrated, the median value in HL rats injected at weaning (2.9) is considerably less than in rats immunized with the same dose of ovalbumin with pertussis adjuvant at the same age (Fig. 13, page 50, Chapter 4). The lack of response to orally-administered LPS may have several explanations: it may not have been absorbed, or inactivated in the gastro-intestinal tract, or the dose may have been inadequate. Adjuvant injected at a site distant from that of the antigen is effective (Farthing, Holt, 1962) so the difference in route of administration of the LPS and the ovalbumin in this experiment does not explain the lack of IgE response.

#### Adjuvant Effect of the Supplementary Feed

It has been shown that intraperitoneal injection of small doses of ovalbumin without adjuvant to weaning HL rats produce a small IgE anti-ovalbumin response (control group, Figure 26 Median value 1.8).

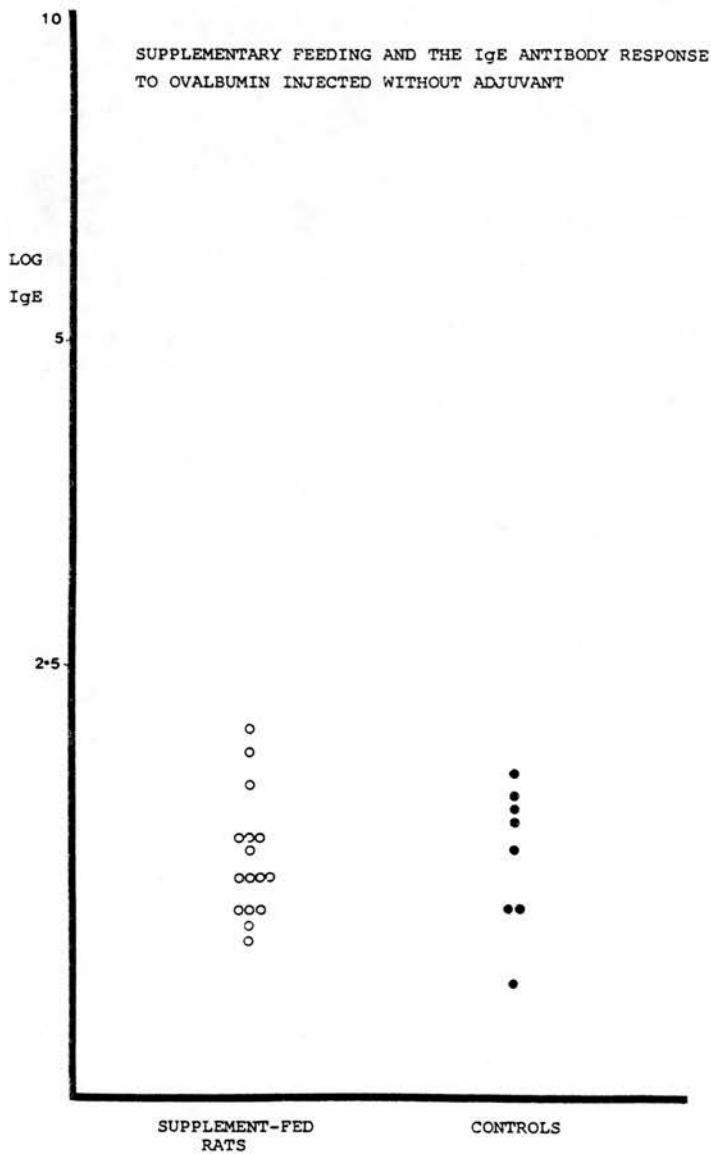
Sera from unimmunized rats gave a maximum value of 1.4 counts bound/counts bound by newborn serum. The effect of supplementary feeds of the cows milk-based preparation on the response to injected ovalbumin without injected adjuvant was examined. Two groups of HL rats from three litters were studied. Sixteen pups received supplementary feeds between three days of age and weaning as previously described (Chapter 3, page 40); A control group of eight pups suckled normally. All the animals were injected intraperitoneally with 10  $\mu$ gs. ovalbumin on day 22 (post-weaning) and with a further dose of 1.0  $\mu$ g on day 56. Blood taken four days later was assayed by PRAST and the secondary IgE antiovalbumin values are shown in Figure 27. Low values were found in both groups and they were not significantly different. Median value in supplemented rats 1.6, range 1.4 - 2.2; median values in controls 1.75, range 1.3 - 2.0.

The experiment was replicated in eight supplement-fed rats and ten litter-mate controls, but using a higher priming dose of injected antigen (100  $\mu$ gs ovalbumin). The secondary IgE values in supplemented rats (median 4.2 range 1.4 - 54.0; Figure 28) were just significantly greater than those control rats (median 2.45 range 1.25 - 5.4). Log transformed data  $\pm$  2.17  $p < 0.05$ . (Mann Whitney U = 26 p NS)(Figure 28).

An antigen dose effect was seen in supplemented rats injected with 10 or 100  $\mu$ gs ovalbumin; the secondary IgE response, assayed in the same batch, was significantly greater with the higher dose (Mann,Whitney U = 28 p  $< 0.05$ ). The difference was not seen in the unsupplemented control rats injected with the two doses of antigen (Mann Whitney, U = 53, p NS). The higher antigen dose was larger than the optimum dose for IgE response to antigen injected with pertussis (Figure 13).

LEGEND TO FIGURE 27:

Secondary IgE antiovalbumin response in supplementary fed and unsupplemented rats injected intraperitoneally with 10  $\mu$ gs ovalbumin without adjuvant in the primary injection. The two groups are not significantly different.

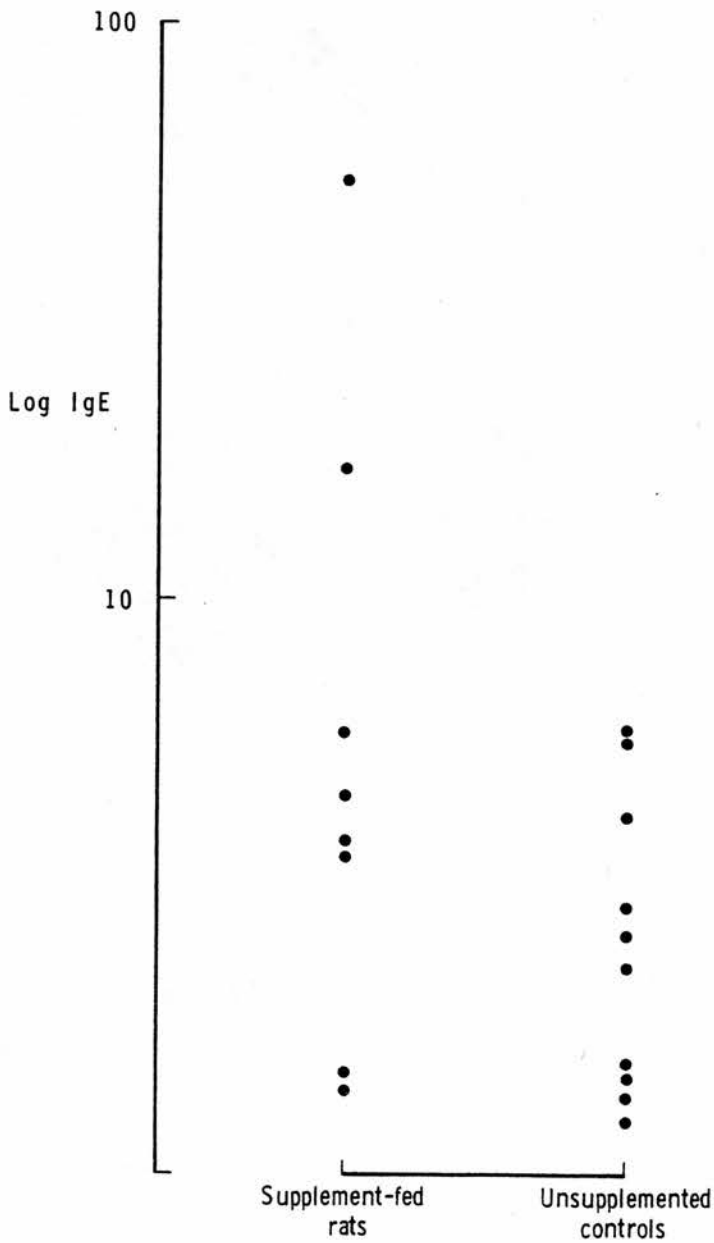




LEGEND TO FIGURE 28:

Secondary IgE antiovalbumin response in supplementary fed rats (median value 4.2 counts bound/counts bound by newborn serum) and unsupplemented controls (median value 2.45) injected intraperitoneally with 100  $\mu$ gs ovalbumin without adjuvant in the primary injection.

(t = 2.17      p < 0.05.)



It is also interesting that, unlike adults, supplement-fed young HL rats consistently produce an IgE response when injected with ovalbumin without adjuvant. Seventeen of twenty-four supplement-fed young rats from both experiments had values higher than those in unimmunized rats ( $> 1.4$  counts bound/counts bound by newborn serum) compared with eight of eighteen unsupplemented rats (Table 8). The difference between the groups is significant ( $\chi^2 = 3.85$   $p = 0.05$ ).

TABLE 8 :

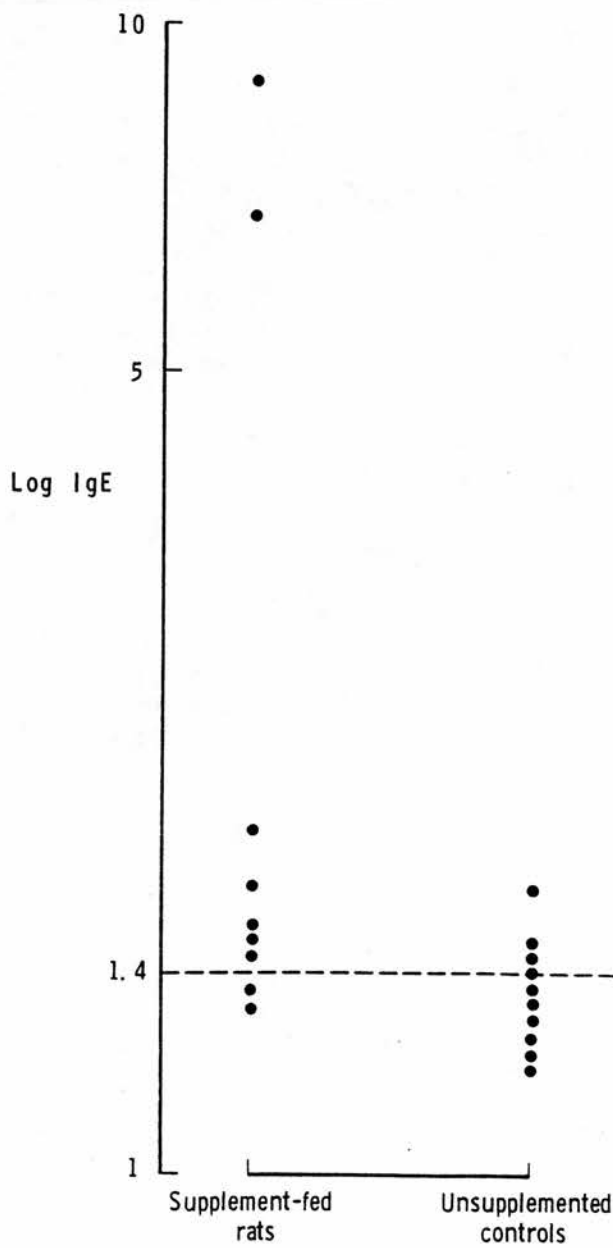
MEDIAN AND RANGE OF IgE ANTIOVALBUMIN RESPONSE IN SUPPLEMENTED AND UNSUPPLEMENTED RATS IMMUNIZED WITH OVALBUMIN INJECTED WITHOUT ADJUVANT.

Injected ovalbumin dose	Supplemented Rats		Unsupplemented Controls	
	<u>n</u>		<u>n</u>	
10 $\mu$ gs	16	1.6(1.4 - 2.2)	8	1.75(1.3-2.0)
100 $\mu$ gs	8	4.2(1.2 - 54)	10	2.45(1.15-5.9)

This finding supports the view that supplementary feeds of cows milk-based preparations have adjuvant effects, increasing the antibody response to injected antigen. Such responses may not be confined to IgE antibody since both IgE and IgG antiovalbumin responses were increased by supplementary feeds (Figures 20 and 21). This interpretation should, however, be regarded with caution because the adjuvant effect was only observed with the higher dose of injected antigen..

LEGEND TO FIGURE 29:

Secondary IgE antiovalbumin antibody in supplement-fed and unsupplemented rats immunized with oral ovalbumin and intraperitoneal pertussis adjuvant are shown. The groups are not significantly different ( $p = 0.1$  Fishers exact test).



### Effects of Supplementary Feeds on the IgE Response to Oral Antigen

Oral administration of ovalbumin in young rats does not usually produce an IgE antiovalbumin response (Chapter 4, page 53). The effect of supplementary feeding on this was investigated. Rat pups from two HL females were studied; nine received supplements of the cows milk-based preparation each day between day 3 and weaning; ten litter-mate controls were suckled normally. At weaning on day 22, the rats were fasted for four hours and gavaged with 10  $\mu$ gs. ovalbumin. They simultaneously received an intraperitoneal injection of  $10^{10}$  Bordetella pertussis. The secondary IgE response was measured at sixty days, and four days after an intraperitoneal injection of 1.0  $\mu$ g ovalbumin (Figure 29). The median value in supplemented rats (1.65 counts bound/ counts bound by newborn serum) was higher than that of the controls (1.45) but not significantly so (Fishers exact test,  $p = 0.1$ ) Both the rats which produced high levels of antibody were supplemented, but the prevalence of response was not significantly greater.

### TOTAL IgE IN SUPPLEMENTARY FED AND CONTROL RATS

Since supplementary feeds of a cows milk preparation to suckling rat pups increase the subsequent IgE and IgG antiovalbumin response, an antigen non-specific effect (Figure 20 ), I investigated, in the same sera of supplementary fed rats and litter mate controls immunized with 1.0  $\mu$ g ovalbumin and pertussis, the IgE concentration, by paper radioimmunosorbent test (Chapter 2, page 30). Doubling dilutions of rat IgE myeloma of known concentration and 1 in 2 dilutions of test sera were incubated with rabbit anti-rat IgE coupled to paper discs. After washing and a further incubation with  $^{125}$ I labelled anti-rat Fc<sup>E</sup>, counts bound were measured and plotted on a standard curve obtained from counts bound by known concentrations of rat IgE myeloma. Results were expressed

in  $\mu\text{g/ml}$ . IgE concentration in the supplementary fed rats (Mean  $0.61 \mu\text{g/ml}$  range  $0.2 - 1.1$ ) did not differ significantly from the controls (Mean  $0.7 \mu\text{g/ml}$  range  $0.34 - 1.4$ ). The values are comparable to those reported by Karlsson (1978) in ten-week old rats of the same strain and using the same method. There is no evidence that the antigen non-specific effect of supplementary feeds is associated with an increase in serum IgE concentrations.

### Discussion

Adjuvant effects of supplementary feeding may provoke IgE responses in susceptible human infants (Lancet editorial, 1979). This hypothesis was explored in the rat; both lipopolysaccharide endotoxin and the supplementary feeds increased the IgE response to injected antigen. Supplementary feeds of cows milk in human infants produce changes in intestinal microflora, producing gram negative bacteria and releasing endotoxin (Bullen et al. 1977). It is not known if such feeds have similar effects in suckling rats and the failure of oral endotoxin to adjuvantize IgE responses awaits explanation. Nevertheless, the data are compatible with an adjuvant effect of supplementary feeds in young rats. Supplementary feeds did not influence the low IgE response to oral ovalbumin, but the experiment was limited to one dose of antigen and immunization by the oral route was found to be unreliable in previous experiments (Chapter 4, page 53). Oral adjuvants may well be less effective than injected ones though IgE responses to ovalbumin have been shown with ovalbumin and Bordetella pertussis given by the intra-gastric route (Bazin and Platteau, 1976).

The effect of supplementary feeds in rats was not one of increasing total IgE as well as specific IgE antibody; serum concentrations of IgE

were similar in supplemented and unsupplemented rats. This is also likely to have been true even if the rats had not been immunized with ovalbumin because Karlsson (1978) has shown that specific IgE antibody (i.e. IgE antiovalbumin) contributes less than 100 ng/ml to total IgE concentration.

In healthy human infants, serum IgE concentrations were greater in bottle-fed babies than in breast-fed ones (Saarinen et al. 1979(b)), and high IgE levels may predict the development of atopic allergies (Kjellman N-IM, 1976). However, infants generically predisposed to atopy do not have higher IgE concentrations with cows milk feeding regimens (Soothill et al. 1976)(Juto, Bjorksten, 1980), so in this respect, do not differ from the supplementary fed rats in my experiments. The findings of Saarinen et al. (1979(b)) may have resulted from the greater prevalence of respiratory and gastrointestinal infection in bottle-fed infants (Gordon et al, 1963)(Chandra, 1979)(Welliver et al.1981), and tend to suggest that high IgE concentrations are an insensitive diagnostic index of atopic allergies.

## Chapter 8

### THE INFLUENCE OF GENETIC AND ENVIRONMENTAL FACTORS ON THE IgE RESPONSES

#### INTRODUCTION

Mature rats show strain related differences in IgE concentration (Karlsson, 1978). Those with the highest levels show greater responsiveness in IgE antibody production (Karlsson, 1978) and hybridization experiments with inbred strains indicate a genetic basis for the differences (Murphey et al. 1974). Within the outbred Hooded Lister strain, considerable interlitter differences in IgE response have been observed which may partly be genetic variation, though this strain generally exhibits high IgE responsiveness in both adult and young rats (Jarrett, Stewart, 1974)(Chapter 4, page 55, Figure 15). There are intralitter differences, too. Genetic variation in rat antibody responsiveness is probably mediated through controlling lymphocyte populations. There are at least two distinct thymic cell populations in rodents separately controlling IgE immunoglobulin class response and the IgE antibody response (Kimoto et al. 1977)(Wanatabe et al. 1977), presumably under separate genetic regulation.

Environmental factors also influence the IgE response; for example, the prevalence of parasitic infestation may increase total IgE and specific IgE antibody (Narva et al. 1981). In addition, early feeding experience in Hooded Lister rat pups influences subsequent IgE antibody response in an antigen non-specific fashion (Chapter 6, Experiments 1 and 2, Figure 20). Outbred rats from different breeding environments might show different patterns of responsiveness. This possibility was investigated in animals from two Hooded Lister rat colonies.

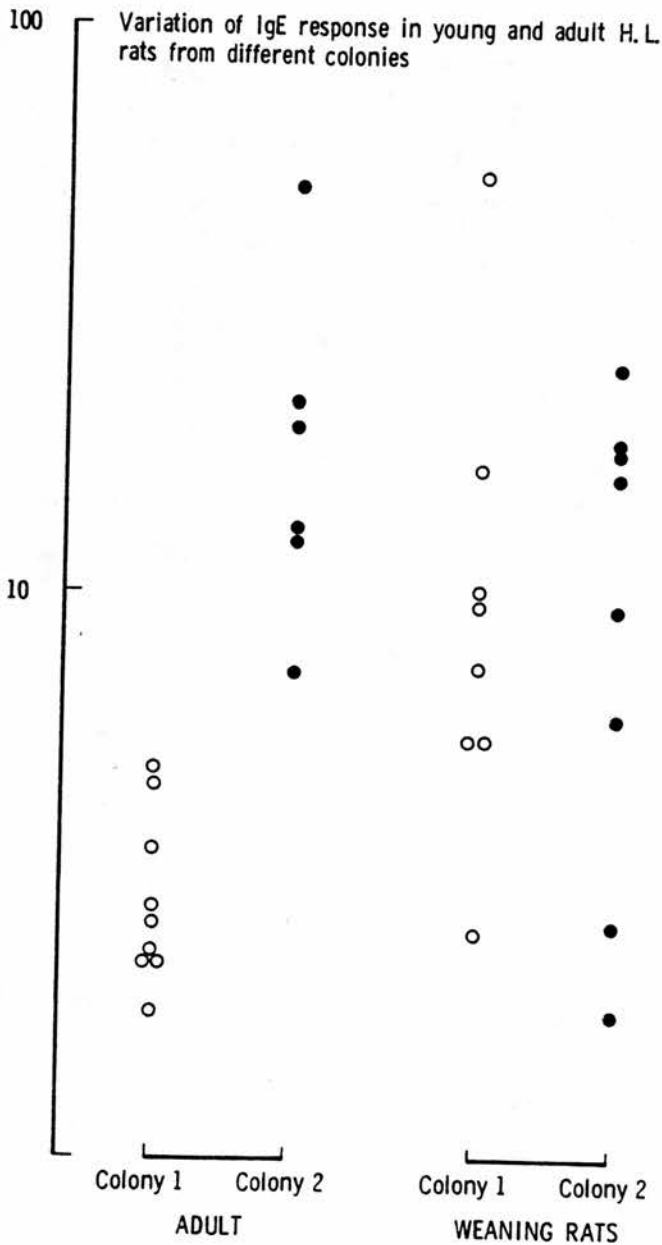
COMPARISON OF IgE RESPONSES IN TWO COLONIES OF HOODED RATS

Hooded Lister rats were purchased from two suppliers - O.L.A.C., Bicester, and Animal Suppliers, London (ASL). Breeding conditions, independently assessed by MRC observers (MRC Laboratory Animals Centre, Carshalton) were less hygienic in the latter - there was a possible risk of parasitic infection. The age related effect of IgE response described previously (Chapter 4, Figure 16) was studied in four groups of rats from the two colonies. The groups of six mature ASL rats (240-300g) and of eight weaning rats chosen from three litters and from the ASL colony, were immunized by intraperitoneal injection with 10 µg ovalbumin and  $10^{10}$  heat-killed Bordetella pertussis. Groups of nine mature and eight weaning rats from OLAC were similarly immunized. The secondary IgE responses were determined in the same batch four days after a second injection of 1.0 µg ovalbumin (Figure 30). Haemagglutinating antiovalbumin was determined in the same sera using ovalbumin coated chromic chloride treated human red cells after absorption of the sera with 50% uncoated erythrocytes. There were no significant differences in the rats immunized at weaning, but mature ASL rats had significantly more IgE antiovalbumin (Figure 30, Colony 2; Median 17, range 7.2 - 53 counts bound/counts bound by newborn serum) than those from OLAC (Colony 1; Median 2.6 range 1.8 - 4.9) (Mann Whitney U = 0  $p < 0.001$ ). These values in adult OLAC rats were significantly less than those in the young rats from either category (Mann Whitney U = 5  $p = 0.006$ ). This finding confirms the greater responsiveness of young rats (Pauwells et al. 1979) though the dose of immunizing antigen was not adjusted for body weight. The low IgE response in mature OLAC rats may have resulted from suppression of IgE by excess antigen, and produced haemagglutinating antibody, or it may have failed to produce any antiovalbumin response. Haemagglutinating antibody titres ( $-\log_2$  titre) were similar in mature rats from both



LEGEND TO FIGURE 30:

Secondary IgE antiovalbumin response in young and adult Hooded rats from two colonies. The difference between adult rats from colony 1 (OLAC - Median value 2.6) and Colony 2 (ASL - Median value 17) is significant,  $p < 0.001$ . No differences between the response in weaning rats.



colonies (Colony 1 mean 11.2 range 3-17. Colony 2 mean 13.6 range 4-17); some antibody response was detected in all but one of the mature animals which confirms that suppression of the IgE response occurred more readily in the animals from Colony 1 (OLAC). The previously described oral immunization experiments (Chapter 4, pages 49 & 53), were repeated with ten weaning rats and eleven mature rats from the OLAC colony. In none of these was IgE antiovalbumin antibody detected (values  $< 1.5$  counts bound/counts bound by newborn serum) and in this respect they did not differ from Hooded rats from Animal Suppliers. IgE antiovalbumin was not detected when serum from these animals was tested by PCA so failure to detect antibody was not the result of IgG antiovalbumin masking the IgE response.

#### GENETIC DIFFERENCES IN THE COLONIES OF HOODED RATS

Inbred and outbred rat strains can be identified by morphological differences in the mandible (Festing, 1976). Possible genetic differences in two colonies of Hooded rat were investigated by Dr.M.W.Festing in twelve mature rats from each colony(250-350g). Results are expressed in arbitrary units of dissimilarity. Values exceeding twelve units are significantly different at the 3% probability level (Chi squared analysis) (Table 9).

The differences between rats from the two suppliers were highly significant, indicating that they are genetically different. The OLAC sample agreed with a reference sample of OLAC Hooded Lister rat previously obtained by Laboratory Animals Centre, Carshalton, and similarly the Animal Suppliers' Hooded rat was similar to the ASL reference sample. However, the Animal Suppliers' rat was not significantly different from the PVG reference sample (Laboratory Animals Centre) and to PVG rats supplied by OLAC( $< 12$  units of difference). The study clearly confirms that not all Hooded rats are identical, and helps to explain observed differences in IgE response.

#### COHABITATION AND CROSS INFECTION BETWEEN TWO HOODED RAT COLONIES.

The higher IgE response in rats from Animal Suppliers may have resulted from prior parasitic infection (Narva, 1981) (Orr, Blair, 1969). Horizontal

TABLE 9:  
COMPARISON OF HOODED RATS FROM OLAC AND ANIMAL SUPPLIERS  
WITH OTHER HOODED RATS

<u>Strain</u>	<u>No.</u>	<u>OLAC</u> test sample	<u>AS</u> test sample
OLAC	12	-	41.7
AS	12	41.7	-
PVG/OLAC	10	21.5	4.5
PVG/Laboratory Animal Centre	10	26.0	4.3

transmission of such infection was encouraged by a cohabitation experiment; eight mature rats from both colonies were caged together for twenty-eight days, and then immunized by intraperitoneal injection with 10 µgs ovalbumin and pertussis adjuvant. The rats appeared to be healthy throughout the study. A second injection of 1.0 µg ovalbumin was given fourteen days later and the secondary IgE antiovalbumin response was determined in serum taken after a further four days. The animals were killed and intestinal contents examined under light microscopy for the presence of parasitic cysts and ova; none were detected. The mean IgE antiovalbumin response in the Animal Suppliers rats (10.6 counts bound/counts bound by newborn serum range 4.0 - 17.3) was significantly higher than the values in OLAC rats (mean 1.67 range < 1.5 - 3.6) (Mann Whitney U = 0,  $p < 0.05$ ). The range of values in rats from two colonies was similar to that observed in non-cohabiting rats (Figure 30), producing some control that cohabitation did not influence the results.

The sera were not examined for specific antiparasitic antibodies but the results showed no evidence of parasitic infection and IgE responses in the less responsive OLAC rats were not increased by cohabitation.

## Discussion

There were significant differences in the IgE antiovalbumin response in mature Hooded rats from separate colonies. Animals immunized at weaning did not show such differences, possibly because of the greater IgE responsiveness observed in young rats (Pauwells et al. 1979) (Chapter 4, Figure 15). Low IgE values observed in one colony of Hooded rat were not the result of immunization failing because haemagglutinating antibody was detected in eight of nine rats. One possible explanation for the different IgE response may have been endemic parasitic infection within one of the colonies, but there is no evidence for this. In addition, breeding conditions are unlikely to influence the relatively high IgE antibody values observed in young rats from either colony. There were significant differences in the morphological characteristics of the Hooded rat colonies, indicating that they were of different strains. ASL Hooded rats have been shown previously to produce high IgE responses (Jarrett, Stewart, 1974). It is likely that inherited differences in controlling lymphocyte populations regulate the antibody response (Wanatabe et al. 1978) though studies of these cellular mechanisms were not performed. The results demonstrate the importance of comparing litter mate groups and of using the same rat colony in investigations of IgE responses. Reaginic antibody responses in humans are also controlled by lymphocyte populations (Buckley, Becker, 1978) (Holt et al. 1981), though environmental factors such as neonatal antigen exposure (Bjorksten, 1980) and infant feeding practise (Mathew et al. 1977) have been shown to influence the IgE response. Such prospective studies of environmental influences in human infants should be interpreted with caution because satisfactory control groups with the same genetic constitution are difficult to attain. The concordance for atopic allergies within families is not great, and there are even differences between identical twins (Wuthrich et al. 1981). However, the observation that supplements of a cows milk-based preparation fed to rat pups influences the subsequent IgE antiovalbumin response, a litter-mate controlled experiment, supports the clinical impression of a similar environmental effect in human infants.

## GENERAL DISCUSSION

Hooded Lister rats were studied, to explore some aspects of human atopic allergy, but there are profound differences from clinical observations in atopic individuals. Rats do not produce cutaneous rashes or allergic rhinitis following exposure to environmental antigen, nor do they spontaneously produce IgE antibody. For them to produce such antibody they usually require injected adjuvant and, unlike atopic patients, this response is of short duration and readily suppressed with continued contact with antigen. The observation that repeated injections of antigen elicit a predominantly IgG antibody response (Jarrett, Stewart, 1974), is more analogous to hyposensitization in the human patient than an allergic response (Lichtenstein et al. 1966). However, the initial antibody response when appropriately immunized with adjuvant is a reaginic one, and this response in the young rat is particularly interesting because it may parallel the origin of atopic disease in human infants.

A number of inherited non-lethal immunodeficiencies are associated with sensitization of atopic patients to several environmental antigens (Soothill, 1976) but there is no evidence for such mechanisms operating in these rats. When first exposed to antigen, genetically predisposed infants may react adversely with an IgE response but there is no evidence that these rats react similarly.

The feeding experiments in rats, designed to parallel supplementary feeding practises in human infants, were not identical to the clinical situation because the supplementary cows milk-derived feeds were given by gastric intubation and they were not interspersed with breast milk throughout the day.

Despite these important differences, considerable similarities have emerged between the allergic response in the two species. Immediate hypersensitivity involving IgE antibody, histamine release from mast cells,

and production of symptoms following antigen challenge has been observed in Hooded rats (Karlsson, 1978). Reaginic responses are regulated by certain lymphocyte populations (Wanatabe et al. 1977), which are controlled by genetic influences; these are responsible for strain differences and individual difference in antibody responsiveness (Kimoto et al. 1977) (Murphey et al. 1974), so that the outbred Hooded Lister strain, which readily produces an IgE response, can be compared with families of atopic individuals. Human reaginic responses are similarly modulated by specific lymphocyte populations (Holt et al. 1981) and imbalances of these have been reported in human atopic infants (Stannegård, Stannegård, 1979) but it is not yet established that they result from genetic or environmental influences.

The influences of environmental factors which certainly contributes to the development of human atopic allergy in genetically predisposed individuals, has not previously been documented in experimental allergy. It is therefore interesting that supplementary cows milk-derived feeds influence IgE responses in the young rat in an antigen non-specific fashion. This finding indicates that sensitization to certain recognised antigens is provoked by dietary modification early in life, as in human infants (Grulee, Sanford, 1936). There is now some evidence that cows milk feeds have adjuvant effects, possibly resulting from alterations in intestinal microflora releasing circulating bacterial endotoxin, but only the IgE response to specific antigen was increased, and serum immunoglobulin E concentration was not affected by the supplementary feeds. The effect of such feeds is not confined to antigen handling by intestinal mucosa because the sensitizing antigen, ovalbumin, was injected. The possibility that adult Hooded rats fed supplements of cows milk respond similarly to young rats was not explored though there is some evidence that supplementary feeds during the suckling period have

an antigen non-specific effect later in life. This is more difficult to explain because adjuvant effects on IgE responses would be unlikely to operate several weeks after the supplements were discontinued. There are receptors on B lymphocytes for the lipid A moiety of endotoxin (Forni, Coutinho, 1978) so that, following the supplementary feeding, more unprimed lymphocytes may be recruited in the primary response and participate in subsequent immunization with the same antigen. Alternatively, cows milk feeds may interrupt the normal maturation of immunity which expresses itself in the rat by modulation and suppression of antibody responses (Pauwells et al. 1979) (Ishizika, Adachi, 1976). There is some evidence that endotoxin prevents tolerance (Ornellas et al. 1970), thus encouraging continued responsiveness to repeated antigen contact, though the observed IgG antibody response in immunized rats argues against systemic hyporesponsiveness as a mechanism of suppression of IgE response.

The effect of different antigen doses on IgE response is less marked in young rats than in adult rats, where large doses of antigen suppress the response. Supplement-fed rats may have persistent unregulated IgE responses into adult life if the supplementary feeds permanently influence antigen handling and IgE production; there is no experimental verification of this hypothesis.

Though some asymptomatic individuals have IgE antibody circulating (Sears et al. 1980), such antibody produces hypersensitivity symptoms in others on exposure to antigen. Symptoms are more frequently observed in bottle-fed infants than breast-fed ones (Mathew et al. 1977) (Chandra, 1979) (Saarinen et al. 1979(a)), so breast milk protects against eczema. The same beneficial effects cannot be confirmed for rat milk because rats do not develop atopic disease. Since IgE-mediated immediate hypersensitivity contributes to human atopic symptoms, IgE mediated anaphylaxis symptoms in rats might be different in supplementary-fed animals and others who



suckled exclusively. Though it is likely that ovalbumin sensitized rats who responded to intravenous challenge with ovalbumin did so because of IgE antiovalbumin antibody, the response was similar in supplementary-fed and unsupplemented rats. Differences may have become apparent if more animals had been studied and if they had not been selected for comparable IgE antibody values. Another explanation for the different response in human infants may be that of other classes of antibody contributing to hypersensitivity reactions (Pepys et al. 1979). It is clear that, in allergic rats, supplementary feeds of cows milk-based preparations have potentially harmful immunological consequences.

Protection against sensitization of the human infant by breast feeding has been confirmed by prospective studies (Mathew, 1977) (Saarinen, 1979)<sup>(a)</sup> but the mechanism of protection is uncertain, and is probably antigen non-specific. In contrast, milk from immunized rats protects suckling offspring by an antigen specific mechanism (Jarrett, Hall, 1979), probably by passive transfer of homologous IgG antibody to the offspring; the protection extends to those offspring who also received supplements of the cows milk-based preparations. It is unlikely that this mechanism operates in the breast-fed human infant, but entirely possible that the maternal IgG antibody which crosses the placenta protects her children against sensitization specifically. It may only protect during the first months of life when immunization, for example against diphtheria, tetanus or salmonella is difficult to achieve (Smith, Eitzman, 1964) (Miller, M.E., 1980) and atopic allergies have not yet become manifest. However, much protection might persist throughout childhood, perhaps by promoting systemic tolerance to sensitizing antigens. Many mothers will have low titres of circulating antibody to cows milk protein (Peterson, Good, 1965) and cows milk is ingested at some time by most infants, yet the prevalence of cows milk protein allergy in atopic infants is surprisingly low (Jakobssen, Lindberg, 1979) (Kletter et al. 1971) (Hill, Davidson, Cameron & Barnes, 1979). Therefore,



in human infants, maternal antibody might induce antigen specific protection against sensitization similar to that in rats suckled by sensitized females. This is likely to be independent of the protective effects of breast milk.

Clinical experience suggests that food allergy differs in several respects from allergy to inhaled or topical antigens; patients exhibit diverse symptoms, latency in response to antigen challenge and symptoms correlate poorly with IgE antibody (Wraith et al. 1979) (Galant et al. 1973) (Foucard, 1973). Nevertheless, some food allergic patients clearly have reaginic antibody to food; they show immediate (type 1) hypersensitivity and pretreatment with oral chromoglycate, which prevents histamine release from mast cells, reduces antigen uptake (Paganelli et al. 1979) and diminishes symptoms (Dahl, Zetterström, 1978). Food allergies are important sources of infant morbidity but their significance appears to decline with increasing age (Jakobssen, Lindberg, 1979). It is not clear why this is but it may reflect differences in antigen handling by the gut, or changes in the immune response to ingested antigen resulting in 'tolerance'. Breast feeding may reduce antigen uptake (Hanson et al. 1977), but food allergy is reported in breast-fed infants - perhaps due to sensitization to small amounts of foreign protein contained in the milk of lactating mothers (Warner, 1980). Experiments with rats have shown antigen is absorbed intact and the absorption is greatest early in life, as in human infants (Warshaw, 1974) (Walker, 1978). Supplementarily-fed rats did not produce IgE antibody to cows milk proteins though this may have been because they were not appropriately immunized with adjuvant, but one characteristic they share with bottle-fed atopic human infants is that they are more readily sensitized to antigen other than cows milk protein, and such ovalbumin-sensitized rats show facilitated antigen absorption following oral ovalbumin challenge (Roberts et al. 1980). The increased uptake of a bystander protein, lactoglobulin, probably

resulted from an ovalbumin-mediated immediate hypersensitivity reaction within intestinal mucosa. Such a mechanism operating in the food-allergic human infant, may increase the risk of sensitization to other food proteins, and suggests a rational basis for prevention of additional sensitization with antigen-avoidance diets.

The conflicting evidence from clinical studies and the experimental data in rats confirm the view that the provocation of human atopic allergies is a complex area; environmental factors such as supplementary infant feeds of cows milk-based preparations contribute to the atopic process but their effects are not just those of exposure to antigen early in life. The protective effects of breast milk are not completely understood nor are they completely preventative, but exclusive breast feeding is, nevertheless, of primary importance in protection of the genetically susceptible infant against atopic allergies.

Appendix 1

CHEMICAL COMPOSITION OF THE COWS MILK-BASED SUPPLEMENT MILK

Cow and Gate Premium Dried Infant Food

Content/100 g

Fat	26.6 g	Vitamin A	630 µg
Protein	14.4 g	Vitamin B <sub>1</sub>	0.53 mg
Lactose	2.7 g	B <sub>2</sub>	0.81 mg
Na	180 mgs	B <sub>6</sub>	0.65 mg
K	480 mgs	B <sub>12</sub>	0.88 µg
Ca	380 mgs	Nicotinic Acid	6.8 mg
Iron	5.2 mgs	Folic Acid	28 µg
Phosphate	250 mgs	Vitamin C	42 mg
Iodine	25 µgs	Vitamin D	8.8 µg
		Vitamin E	8 mg
		Biotin	25 µg

Casilan

Content/100 g

Protein (Calcium Caseinate)	90 g
Fat	1.8 g
Mineral Salts	3.8 g
Calcium	1.2 g

Prosparol

50% Arachis oil in aqueous suspension  
Calories 4.5 c/ml.

(1) 1M Na OH                      40 g Na OH                      1 litre deionized H<sub>2</sub>O.

(2) Carbonate/Bicarbonate buffer pH 9.0

Stock Solutions: A. Bicarbonate  $\text{NaHCO}_3$  42 g 1 litre

B. Carbonate  $\text{Na}_2\text{CO}_3$  13.25 g 250 ml

0.5M NaH CO<sub>3</sub> pH 9.0 Mix carbonate with bicarbonate in proportions 1:9 V/V.

(3) 0.1 NaH CO<sub>3</sub> NaCL 0.5M pH 9.0 Dilute 0.5M NaH CO<sub>3</sub> 1:5  
NaCL 29.22 g 1 litre

(4) 0.1M Tris HCL buffer pH 8.0      12.11 g Tris. 50 ml. 1M HCL.      1 litre

(5)   0.1M Acetate buffer     pH 4.0        13.6 g sodium acetate trihydrate,  
    5.73 glacial acetic acid                ) 2 litres

(6) 0.5M Phosphate buffer pH 7.4

Stock Solutions:      A. Anhydrous  $\text{Na}_2\text{HPO}_4$  142g  
                                  B.  $\text{KH}_2\text{PO}_4$  34 g      ) 2 litres

(7) Incubation buffer                      dilute 0.5M Phosphate buffer 1:10    2 litre  
NaCL         5.84 g  
Tween 20    1%    2 ml (Sigma Chemicals)  
Bovine serum albumin 600 mg. (Miles Laboratories)

(8) Diluent buffer	NaCL	90 g	1 litre
	Bovine serum albumin		10 g
	Human IgG (Kabi)		100 mg
	Na Azide		1 g

(9) Phosphate buffer Saline      pH 7.4

NaCL	80 g	1 litre
NaH PO <sub>4</sub>	12 H <sub>2</sub> O	28.1 g
KCL		2 g

(10) 0.15M NaCL	88.25 g	1 litre
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BIBLIOGRAPHY

- ABRAHAMSON, D.R., POWERS, A., RODEWALD, R.: Science, 1979. 206(4418) 567.  
'Intestinal absorption of immune complexes by neonatal rats: a route of antigen transfer from mother to young'.
- AMMANN, A.J., STEHM, E.R.: Proc.Roy.Soc.exp.Biol.Med., 1966. 122 1098.  
'Immune globulin levels in colostrum and breast milk, and serum from breast and formula fed newborns'.
- ANDERSON, K.J., McLAUGHLAN, P., DEVEY, M.E., COOMBS, R.R.A.: Clin.Exp.Immunol., 1979. 35 454. 'Anaphylactic sensitivity of guinea pigs drinking different preparations of cows milk and infant formulae'.
- ANDRE, C., HEREMANS, J.F., VAERMAN, J.P., CAMBIASO, C.-L.: J.Exp.Med., 1975. 142 1509. 'A mechanism for the induction of immunological tolerance by antigen feeding'.
- ATHERTON, D.J., SEWELL, M., SOOTHILL, J.F., WELLS, R.S., CHILVERS, C.E.D.: Lancet, 1978. 1 401. 'A double blind controlled crossover trial of an antigen avoidance diet in atopic eczema'.
- BAZIN, H., PLATTEAU, B.: Immunology, 1976. 30 679. 'Production of circulating reaginic (IgE) antibodies by oral administration of ovalbumin to rats'.
- BENACERAFF, B., McDEVITT, H.O.: Science, 1972. 175 273.  
'Histocompatibility-linked immune response genes'.
- BENNICH, H.H., ELLERSON, J.R., KARLSSON, J.: Immunology Reviews, 1978. 41 261. 'Evaluation of basic serum IgE levels and the IgE antibody response in the rat by radioimmuno assays'.
- BINAGHI, R.A., BENACERAFF, B.: J.Immunol., 1964. 92 920. 'The production of anaphylactic antibody in the rat'.
- BJORKSTEN, F., SUONIEMI, I., KOSKI, V.: Clin.Allergy, 1980. 10 585.  
'Neonatal birch pollen contact and subsequent allergy to birch pollen'.
- BJORKSTEN, F., SOTARINEN, U.M.: Lancet, 1978. ii. 624.  
'IgE antibodies to cows milk in infants fed breast milk and milk formulae'.
- BLOCH, K.J., BLOCH, D.B., STEARNS, M., WALKER, W.A.: Gastroenterology, 1979. 77 1039. 'Intestinal uptakes of macromolecules VI. Uptake of protein antigen in vivo in normal rats and rats subjected to mild systemic anaphylaxis'.
- BOMFORD, R.: Immunology, 1981. 44 187. 'The adjuvant activity of fatty acid esters. The rôle of acyl chain length and degree of saturation'.

- BORTHISTLE, B., KUBO, R.T., BROWN, W.B.: J.Immunol., 1977. 119 471.  
'Studies on receptors for IgG anepithelial cells of the rat intestine'.
- BRAMBELL, F.W.R., HEMMINGS, W.A.: J.Physiol., 1949. 108 177.  
'The passage into the embryonic yolk-sac cavity of maternal plasma proteins in rabbits'.
- BRAMBELL, F.W.R.: Lancet, 1966. 2 1087. 'The transmission of immunity from mother to young and the immunoglobulins'.
- BROSTOFF, J., CARINI, C., WRAITH, D.G., PAGANELLI, R., LEVINSKY, R.J.:  
'The Mast Cell' (editors J.Pepys and A.M. Edwards), Pitman, London. 1979. p.380.  
'Immune complexes in Atopy'.
- BUCKLEY, R.H., DEES, S.C.: New England J.Med., 1969. 281 465.  
'Correlation of milk precipitins with IgA deficiency'.
- BUCKLEY, R.H., BECKER, W.G.: Immunology Reviews, 1978. 41 288-314.  
'Abnormalities in the regulation of human IgE synthesis'.
- BULLEN, C.L., TEARLE, P.V., STEWARD, M.G.: J.Med.Microbiol., 1977. 10 404.  
'The effect of humanised milks and supplemented breast-feeding on the faecal flora of infants.
- CHANDRA, R.K.: Acta.Paed.Scand., 1979. 68 691. 'Prospective studies of the effect of breast feeding on the incidence of allergy and infection'.
- CHURCH, J.A., FRENKEL, L.D., WRIGHT, D.G., BELLANTI, J.A.: J.Paed., 1976. 88 982. 'T lymphocyte dysfunction, hyperimmunoglobulinaemia E, recurrent bacterial infections and defective neutrophil chemotaxis in a Negro child'.
- COCA, A.F., COOKE, R.A.: J.Immunol., 1923. 8 163. 'On the classification of the phenomena of hypersensitiveness'.
- COOKE, R.A., VANDERVEER, A.: J.Immunol., 1916. 1 216.  
'Human sensitization'.

- DAHL, R., ZETTERSTRÖM, O.: Clinical Allergy, 1978. 8 419.  
'The effect of orally administered sodium chromoglycate on allergic reactions caused by food allergens'.
- DYMSZA, H.A., CZAKI, D.M., MILLER, S.A.: J.Nutrition, 1964. 84 100.  
'Influence of Artificial diet on Weight gain and body composition of the Neonatal Rat'.
- EASTHAM, E.J., LICHUSCO, T., BRODY, M.I., WALKER, W.A.: J.Paed, 1978. 93 561. 'Role of the immature intestine on protein permeability'.
- EVANS, R.W., FERGUSON, D.M., ALLARDYCE, R.A., TAYLOR, B.: Lancet, 1981. i 1340. 'Maternal diet and infantile colic in breast fed infants'.
- FARTHING, J.R., HOLT, L.B.: J.Hygiene. Camb., 1962. 60 411.  
'Experiments designed to determine the mechanism of the adjuvant activity of Gram-negative organisms upon antibody production'.
- FESTING, M.F.W.: Nature, 1976. 263(5574) 230. 'Phenotypic variability of inbred and outbred mice'.
- FIRER, M.A., HOSKING, C.S., HILL, D.J.: BMJ., 1981. 283 693.  
'Effect of antigen load on the development of milk antibodies in infants allergic to milk'.
- FORNI, L., COUTINHO, A.: Nature, 1978. 273 304. 'Receptor interactions on the membrane of resting and activated B cells'.
- FOUCARD, T.: Acta Paediatrica Scandinavica, 1973. 62 633. 'A follow-up study of children with asthmatoïd bronchitis: Skin test reactions and IgE antibodies to common allergens'.
- GALANT, S.P., BULLOCK, J., FRICK, O.L.: Clin.Allergy, 1973. 3 363.  
'An immunological approach to the diagnosis of food sensitivity'.
- GLASER, J., JOHNSTONE, D.E.: J.Amer.Med.Ass., 1953. 153 620.  
'Prophylaxis of allergic disease in Newborns'.
- GOOD, R.A., PAPERMASTER, B.W.: Advances in Immunol., 1964. 4 1.-115.  
'Ontogeny and Phylogeny of adaptive immunity'.
- GORDON, J.E., CHITKARA, I.D., WYAN, J.B.: Amer.J.Med.Science, 1963. 346 129. 'Weaning Diarrhoea'.
- GROSS, S.J., BUCKLEY, R.H.: Lancet, 1980. ii 543. 'IgA saliva of breast fed and bottle fed infants'.
- GRULEE, C.S., SANFORD, H.N.: J.Paed., 1936. 9 223. 'The influence of breast feeding and artificial feeding in infantile eczema'.
- GRUSKY, F.L., COOKE, R.E.: Paediatrics, 1955. 16 763.  
'Gastrointestinal absorption of unaltered protein in normal infants recovering from diarrhoea'.
- GWYNN, C.M., MORRISON-SMITH, J., LEAN, G.L., STANWORTH, D.R.: Clinical Allergy, 1979. 9 119. 'IgE and IgG<sub>4</sub> subclasses in atopic families'.

- HALL, W.G.: Science, 1975. 190 1313. 'Weaning and growth of artificially reared rats'.
- HAMOAKA, T., KATZ, D.H.: J.Immunol., 1973. 3 1554. 'Cellular site of action of various adjuvants in antibody responses to hapten carrier conjugates'.
- HANSON, L.A., AHLESTEDT, S., CARLSSON, B., FALLSTROM, S.P.: Int.Arch.Allergy & Applied Immunol., 1977. 54 457. 'Secretory IgA antibodies against cows milk proteins in human milk and their possible effect in mixed feeding'.
- HEIRD, W.C., HANSEN, I.H.: Paed.Research, 1977. 2 406. 'Effect of colostrum on growth in intestinal mucosa'.
- HEMMINGS, W.A., WOOD, C.: IRCS. Medical Science, 1975. 3 242. 'Protein absorption by the gut of the suckling rat following oral immunization'.
- HIDE, D.W., GUYER, B.M.: Arch.Dis.in Childhood, 1981. 56 172. 'Clinical manifestation of allergy related to breast and cows milk feeding'.
- HILL, D.J., DAVIDSON, G.P., CAMERON, D.J., BARNES, G.L.: Acta.Paed.Scand., 1979. 68 847. 'The spectrum of cows milk allergy in childhood. Clinical, gastro-enterological and immunological studies'.
- HO, May-Kin., MORSE, S.I., KONG, A.S.: J.Exp.Med., 1981. 153 75. 'Studies on a new lymphocyte mitagen from Bordetella pertussis'.
- HOLT, P.G., TURNER, K.J., HOLT, B.G.: Clinic.Exp.Immunol., 1981. 45 523. 'In vitro synthesis of IgE by human peripheral blood leucocytes. ii) modulation of IgE B cell activity by isotype-specific regulatory cells'.
- HUGGINS, K.G., BROSTOFF, J.: Lancet, 1975. ii 148. 'Local production of specific IgE antibodies in allergic rhinitis patients with negative skin tests'.
- ISHIZIKA, K., ADACHI, T.: J. Immunol., 1976. 117 40. 'Generation of specific helper cells and suppressor cells in vitro for the IgE and IgG antibody responses'.
- IYNGKARAN, N., DANS, K., ROBINSON, M.J., BOEY, C.G., SUMITHRAN, E., YADAV, M., CAM, S.K., PUTHUCHEARY, S.D.: Arch.Dis.Child., 1979. 54 39. 'Cows milk protein-sensitive enteropathy'.



- JAKOBSEN, J., LINDBERG, T.: Lancet, 1977. ii 437.  
'Cows milk as a cause of infantile colic in breast fed infants'.
- JAKOBSEN, J., LINDBERG, T.: Acta.Paed.Scand., 1979. 68 853.  
'A prospective study of cows milk protein intolerance in Swedish infants'.
- JARRETT, E.E.E., STEWART, D.C.: Immunology, 1974. 27 365.  
'Rat IgE Production I. Effect of dose of antigen on Primary and Secondary reaginic antibody responses'.
- JARRETT, E.E.E., Lancet, 1977. ii 223. 'Activation of IgE a regulatory mechanisms by transmucosal absorption of antigen'.
- JARRETT, E.E.E., HAIG, D.M., McDOUGALL, W., McNULTY, E.: Immunology, 1976. 30 671. 'Rat IgE Production II. Primary and booster reaginic antibody responses following intradermal or oral immunization'.
- JARRETT, E.E.E., HALL, E.: Nature, 1979. 280 145. 'Selective suppression of IgE antibody responsiveness by maternal influence'.
- JARRETT, E.E.E., STEWART, D.C.: Immunol., 1972. 23 749.  
'Potentiation of rat IgE antibody by helminth infection'.
- JARRETT, E.E.E., MACKENZIE, S., BENNICH, H.: Nature, 1980. 283 302.  
'Parasite induced non-specific IgE does not protect against allergic reactions'.
- JARRETT, E.E.E., HALL, E., KARLSSON, I., BENNICH, H.: Clin.Exp.Immunol., 1980. 39 183. 'Adjuvants in the induction and enhancement of rat IgE responses'.
- JOHNSTONE, D.E., DUTTON, A.M.: N.Eng.J.Med., 1966. 274 715.  
'Dietary prophylaxis of Allergic disease in Children'.
- JUTO, R., STANNegÅRD, O.: J.Allergy & Clin.Immunology, 1979. 64 38.  
'T lymphocytes and blood eosinophils in early infancy in relation to heredity for allergy and type of feeding'.
- JUTO, R., BJORKSTEN, B.: Clin.Allergy, 1980. 10 593. 'Serum IgE in infants and influence of type of feeding'.
- KARLSSON, T., ELLERSON, J.R., HAIG, D.M., JARRETT, E.E.E., BENNICH, H.H.: Scand.J.Immunol., 1979. 9 229. (a) 'A radioimmunoassay for evaluation of the IgE and IgG antibody responses in the rat'.
- KARLSSON, T.; 'Immunoglobulin E and the Mast Cell'. Ph.D. Thesis. University of Uppsala, 1978.
- KARLSSON, T., ELLERSON, J.R., DAHLBOM, I., BENNICH, H.: Scand.J.Immunol., 1979. 9 217. (b) 'Analysis of the serum IgE levels in non-immunized rats of various strains by a radioimmunoassay'.
- KATZ, D.W.: Clin.Allergy, 1979. 9 609. 'New concepts concerning the clinical control of IgE synthesis'.
- KIKKAWA, Y., KAMIMURA, K., MAMAJIMA, T.: Paed., 1973. 51 690.  
'Thymic alymphoplasia with hyper IgE globulinaemia'.

- KILSHAW, P.J., SLADE, W.: Clinical & Experimental Immunol., 1980. 41 575. 'Passage of ingested protein into the blood during gastro-intestinal hypersensitivity reactions: experiments in the pre-ruminant calf'.
- KIMOTO, M., KISHIMOTO, I., NOGUCHI, S., WANATABE, T., YAMAMURA, Y.: J.Immunology, 1977. 118 3 840. 'Regulation of antibody response in different immunoglobulin classes'.
- KJELLMAN, N-IM.: Acta.Paed.Scand., 1976. 65 465. 'Predictive value of high IgE levels in children'.
- KLETTER, B., GERY, S., FREIER, S., NOAH, Z., DAVIES, M.: Clin.Allergy, 1971. 1 249. 'Immunoglobulin E antibodies to milk protein'.
- KOFLER, R., WICK, G.: J.Immun.Methods 1977. 16 201. 'Some methodological aspects of the chromium chloride method for coupling antigen to erythrocytes'.
- LANCET EDITORIAL: Food Allergy. 1979. i 249. 'Oral encounters with antigen' 1981. i. 702.
- LECCE, J.G.: Biologia Neonatorum, 1966. 9 50. 'Absorption of macromolecules by neonatal intestine'.
- LEVINE, B.B., VAZ, N.M.: Int.Archives Allergy, 1970. 39 151. 'Effect of combination of inbred strain, antigens and antigen dose on immune responsiveness and reagin production in the mouse'.
- LICHTENSTEIN, L., NORMAN, P., WINKENWERDER, W., OSLER, A.: J.Clin.Invest., 1966. 45 1126. 'In vitro studies of human ragweed allergy: changes in cellular and humoral activity associated with specific desensitization'.
- LUCKEY, T.D., MENDE, T.J., PLEASANTS, J.: J.Nutrition, 1954. 54 345. 'The physical and chemical characterisation of rat milk'.
- LYNSTER, R.L.J.: J.Dairy Research, 1972. 39 279. 'Quantitation of the proteins in cows milk'.
- MACKENZIE, D.D.S.: Amer.J.Physiol., 1972. 223 1286. 'Selective uptake of immunoglobulin by the proximal intestine of suckling rats'.
- MANN, H.B., WHITNEY, D.R.: Annals of mathematical statistics, 1947. 18 52.
- MARSH, D.G., BIAS, W.B., HSU, S.H.: Science, 1973. 179 691. 'Association of the HL-A7 Cross reacting group with a specific reaginic antibody response in Allergic Man'.
- MATHEW, D.J., TAYLOR, B., NORMAN, A.P., TURNER, M.W.; SOOTHILL, J.F.: Lancet, 1977. i 321. 'Prevention of eczema'.
- MATHEWS, J.D., WHITTINGHAM, S., MACKAY, I.R., MALCOLM, L.A.: Lancet, 1972. ii. 675. 'Protein supplementation and enhanced antibody producing capacity in New Guinea schoolchildren'.

- McGHEE, J., MICHALEK, S., GHANTA, V.K.: Immunochemistry, 1975. 12 817. 'Rat immunoglobulins in serum and secretions: Purification of Rat IgM IgA and IgG and their quantitation in serum, colostrum, milk and saliva'.
- MESSER, M., THOMAS, E.B., TERRASA, A.G., DALLMAN, P.R.: J.Nutrition, 1969. 98 404. 'Artificial feeding of infant rats by continuous gastric infusion'.
- MILLER, M.E.: In Allergic Diseases of Infancy, Childhood and Adolescence. (Editors, Bierman and Pearlman.) W.B. Saunders Co., 1980. p.30. 'Antibody responses of the neonate'.
- MILLER, S.A., CZAKI, D.M.: Biol.Neonate, 1967. 11 197. 'The influence of dietary osmolarity on survival in the neonatal rat'.
- MIYACHI, S., LESSOF, M.H., KEMEMY, D.M., GREEN, L.A.: International Archs. Allergy Appl.Immunol., 1979, 58 160. 'Comparison of the Atopic background between Allergic and Non-Allergic Beekeepers'.
- MORRIS, B., MORRIS, R.: J.Physiol., 1976. 255 619. 'Quantitative assessment of the transmission of labelled proteins by the proximal and distal regions of the small intestine of young rats'.
- MORAG, M.: Laboratory Animals, 1970. 4 2 259. 'Estimation of milk yield in the rat'.
- MORRISON, D.C., RYAN, J.L.: Advances in Immunology, 1979. 25 293-450.
- MORRISON, D.C., ULEVITCH, R.J.: Am.J.Pathol., 1978. 93 526-617. 'The effects of bacterial endotoxins on host mediation systems: A Review'.
- MORRISON-SMITH, J.: BMJ., 1961. 1 711. 'Prevalence and natural history of asthma in schoolchildren'.
- MORRISON-SMITH, J., SPRINGETT, V.H.: Clin.Allergy, 1979. 9 153. 'Atopic disease and month of birth'.
- MURPHEY, S.M., BROWN, S., MIKLOS, N., FIREMAN, P.: Immunology, 1974. 27 245. 'Reagin synthesis in inbred strains of rats'.
- NARVA, Y., MILLER, H.R.P., HALL, E., JARRETT, E.E.E.: Immunology, 1981. 44 119. 'Adoptive transfer of total and parasite specific IgE responses in rats infected with *Nippostrangylus Brasiliensis*'.

- OLSON, P.R., WEIBLEN, E.J., O'LEARY, J.J., MOSCOWITZ, A.J., McCULLOUGH, J.: Vox Sanguinis, 1976. 30 149. 'A Simple technique for inactivation of IgM using Dithiothreitol'.
- ORNELLAS, E.P., SANFILLIPPO, F., SCOTT, D.W.: European J.Immunol., 1970. 19 387. 'Reversal of tolerance in rats at the B cell level by endotoxin'.
- ORR, T.S.C., BLAIR, A.M.J.N.: Life Science, 1969. 8 1073. 'Potentiated reagin response to egg albumin in *Nippostrongylus Brasiliensis* infected rats'.
- ORREN, A., DOWDLE, B.B.: International Archives. Allergy and Applied Immunol., 1975. 48 224. 'The effects of sex and age on serum IgE concentrations in three asthmatic groups'.
- OUCHTERLONY, O.: Immunological Methods (Ed. Ackroyd, J.F.) 1964. Blackwell Scientific Publications. Oxford. 'Gel diffusion methods'.
- OVARY, Z.: J.Immunol., 1958. 81 355. 'Passive cutaneous anaphylaxis in the mouse'.
- Int.Arch.Allergy, 1952. 3 293. 'Cutaneous anaphylaxis in the albino rat'.
- PAGANELLI, R., LEVINSKY, R.J., BROSTOFF, J., WRAITH, D.G.: Lancet, 1979. i 1270. 'Immune complexes containing food proteins in normal and atopic subjects after oral challenge and effect of Sodium chromoglycate on antigen absorption'.
- PASSWELL, J.H., STEWARD, M.W., SOOTHILL, J.R.: Clin.Exp.Immunol., 1974. 17 491. 'The effects of protein malnutrition on macrophage function and the amount and affinity of antibody responses'.
- PAUWELLS, R., BAZIN, H., PLATTEAU, B., VAN DER STRAETON, M.: Immunology, 1979, 36 145. 'The effect of age on IgE production in rats'.
- PEPYS, J., PARISH, W.E., STENIUS-AARNIALA, B., WIDE., L.: Clin.Allergy 1979. 9 645. 'Clinical correlations between long-term (IgE) and short-term (IgG S-TS) anaphylactic antibodies in atopic and "non-atopic" subjects with respiratory allergic disease'.
- PERINI, A., MOTA, I.: Immunology, 1973. 25 297. 'The production of IgE and IgG<sub>1</sub> antibodies in guinea pigs immunized with antigen and bacterial lipopolysaccharides'.
- PETERSON, R.D.A., GOOD, R.A.: Paediatrics, 1965. 31 209. 'Antibodies to cows milk proteins - their presence and significance'.
- PHILLIPS-QUAGLIATA, J.M.: Cellular Immunology, 1972. 4 116. 'Cellular Events in Tolerance i) Induction and loss of tolerance in neonatal and adult rats'.

- PITTARD, W.B., BILL, K.: Clin.Immunol.and Immunopathology, 1979. 13 430. 'Differentiation of cord blood lymphocytes into IgA producing cells in response to breast milk stimulatory factor'.
- PLATTS-MILLS, T.A.E., SNAJDR, M.J., ISHIZAKA, K., FRANKLAND, A.W.: J.Immunol., 1978(4) 120 1201. 'Measurement of IgE antibody by an antigen binding assay: correlation with PK activity and IgG and IgA antibodies to allergens'.
- REDDY, R.R., DONKER, J.D., LINNERUD, A.C.: J.Dairy Science, 1964. 47 554. 'Rates of milk secretion between nursing intervals of 3,6,9 and 12 hours using Sprague Darsley rats'.
- REED, C.E., BRENNER, M., LOCKEY, S.D., ENTA, M., MAKINO, S., CARR, R.H.: J.Allergy & Clin. Immunol., 1972. 49 175. 'On the mechanisms of the adjuvant effect of Bordatella Pertussis vaccine'.
- ROBERTS, S.A., FREED, D.I.J.: Lancet, 1977. ii 1131. 'Neonatal IgA secretion enhanced by breast feeding'.
- ROBERTS, S.A., GREGORY, B.J.: Laboratory Animals, 1980. 14 323. 'Facultative Pasteurella ophthalmitis in Hooded Lister rats'.
- ROBERTS, S.A., REINHARDT, M.C., PAGANELLI, R., LEVINSKY, R.J.: Clin.Exp.Immunol., 1981. 45 131. 'Specific antigen exclusion in rats allergic to food proteins'.
- RODEWALD, R.: J.Cellular Biol., 1973. 58 189. 'Intestinal transport of antibodies in the newborn rat'.
- ROTHBERG, R.M.: J.Paed., 1969 75 391. 'Immunoglobulin and specific antibody synthesis during the first weeks of life in premature infants'.
- SAARINEN, U.M., KAJOSAARI, M.: Lancet, 1980. i 166. 'Does dietary elimination in infancy prevent or only postpone a food allergy?'
- SAARINEN, U.M., KAJOSAARI, M., BACHMAN, A., SIIMES, M.A.: Lancet, 1979.<sup>(a)</sup> ii 163. 'Prolonged breastfeeding as prophylaxis for atopic disease'.
- (b)
- SAARINEN, U.M., BJORKSTEN, F., KNEKT, P., SIIMES, M.A.: Clin.Allergy 1979. 9 339. 'Serum IgE in healthy infants fed breast milk or cows milk-based formulas'.
- SADOWSKI, P.L., ROBBINS, K.E., MUNOZ, J.J.: IRCS Medical Science. Immunology & Allergy, 1979. 7 612. 'Stimulation of IgE by Pertussigen'.
- SCHLESINGER, J.J., COVELLI, H.D.: Lancet, 1977. ii 529. 'Evidence for Transmission of lymphocyte responses to tuberculin by Breast-feeding'.

- SCHWARTZ, H.J., LESKOWITZ, S., LOWELL, F.C.: J.Allergy, 1968. 42 169.  
'Evidence for an abnormality of mucosal surfaces in atopic subjects'.
- SEARS, M.R., CHOW, C.M., MORSETH, D.J.: Clin.Allergy, 1980. 10 423.  
'Serum total IgE in normal subjects and the influence of a family history of allergy'.
- SMITH, C.W.: J.Paediatrics, 1973. 82 1082. 'Host resistance factors in human milk'.
- SMITH, M.W., BURTON, K.A., MUNN, E.A.: In Protein transmission through lung membranes. (Editor W.A. HEMMINGS) Elsevier/North Holland Biochemical Press, 1979. p.197.  
'Vacuolation and non-specific protein transport by newborn pig intestine'.
- SMITH, R.T., EITZMAN, D.V.: Paediatrics, 1964. 33 163.  
'The development of the immune response- characterization of the response of the human infant and adult to immunization with Salmonella vaccines'.
- SOOTHILL, J.F., STOKES, C.R., TURNER, M.W., NORMAN, A.D., TAYLOR, B.: Clinical Allergy, 1976. 6 305. 'Predisposing factors and the development of reaginic allergy in infancy'.
- SOOTHILL, J.F.: Proc.Royal Soc.Med., 1976. 69 439. 'Some intrinsic and extrinsic factors predisposing to allergy'.
- STANNEGÅRD, I-L., STANNEGÅRD, O.: Scand.J.Immunol., 1977. 6 1225.  
'Increased sensitivity of lymphocytes from atopic individuals to histamine-induced suppression'.
- Clin.Allergy, 1979. 9 637-642. 'In vitro differences between the lymphocytes of normal subjects and atopics'.
- STAVITSKY, A.B.: Immunological Methods. (Ed.Ackroyd, J.F.) 1964. p.363. Blackwell Scientific Publications, Oxford.  
'Haemagglutination and haemagglutination inhibition reactions'.
- STOLIAR, O.A., KANIECKI-GREEN, E., PELLEY, P.R., KLAUS, M.H., CARPENTER, C.C.J.: Lancet, 1976. i 1258. 'Secretory IgA against enterotoxins in breast milk'.
- SWARBRICK, E.T., STOKES, C.R., SOOTHILL, J.F.: Gut, 1979. 20 121.  
'Absorption of antigens after oral immunization and the simultaneous induction of specific systemic tolerance'.
- TADA, T.: Progress in Allergy, 1975. 19 122. 'Regulation of reaginic antibody formation in animals'.
- TADA, T., OKUMURA, K.: J.Immunol., 1970. 106 1002.  
'Regulation of homocytotropic antibody formation in the rat. Feed back regulation by passively administered antibody'.



- THOMAS, J.C., PARROTT, D.M.V.: Immunology, 1974. 27 631.  
'The induction of tolerance to a soluble protein antigen by oral administration'.
- THOMAS, W.R., WATKINS, M.C., ASHERSON, G.L.: Immunology, 1978. 35 41.  
'Reaginic antibody to contact sensitizing agents; occurrence of cells which suppress IgG and not reaginic responses'.
- TURNER, K.J., ROSMAN, D.L., O'MAHONY, J.: International Arch.Allergy & Applied Immunol., 1974. 47 650. 'Prevalence and familial association of Atopic disease and its relationship to serum IgE levels in 1061 schoolchildren and their families'.
- VIANDER, M., KOIVIKKO, A.: Clin.Allergy, 1978. 8 387.  
'The seasonal symptoms of hyposensitivity and untreated hay fever patients in relation to birth pollen counts; correlations with nasal sensitivity, prick tests and RAST'.
- WALKER, W.A., WU, M., ISSELBACHER, K.J., BLOCH, K.J.: Gastro.ent., 1975(a) 69 1225. 'Intestinal uptake of macromolecules'.
- WALKER, W.A.: Arch.Dis.Child., 1978. 53 527. 'Antigen handling by the gut'.
- WALKER, W.A., ISSELBACHER, K.J.: Gastroenterology, 1974. 67 531.  
'Uptake and transport of macromolecules by the intestine'.
- WALKER, W.A., WU, M., ISSELBACHER, K.J.: J.Immunol., 1975.<sup>(b)</sup> 115 854.  
'Intestinal uptake of macromolecules III. Studies on the mechanism by which immunization interferes with antigen uptake'.
- WANATABE, N., KOJIMA, S., SHEN, F., OVARY, Z.: J.Immunol., 1977. 118 2. 485. 'Suppression of IgE antibody production in SJL mice'.
- WARNER, J.O.: Clinical Allergy, 1980. 10 133. 'Food allergy in fully breast-fed infants'.
- WARSHAW, A.L., WALKER, W.A., ISSELBACHER, K.J.: Gastroenterology, 1974. 66 987. 'Protein uptake by the intestine: evidence for absorption of intact macromolecules'.
- WELLIVER, R.C., WONG, D.T., SUN, M., MIDDLETON, E., VAUGHAN, R.S., OGRA, P.L.: New Eng.J.Med., 1981 305 841. 'The development of respiratory syncytial virus-specific IgE and the release of histamine in nasopharyngeal secretions after infection'.
- WIDDOWSON, E.M., COLOMBO, V.E., ARTAVANIS, C.A.: Biol.Neonate, 1976. 28 272. 'Changes in the organs of pigs in response to feeding for the first 24 hours after feeding. II. The digestive tract'.
- WIDE, L., BENNICH, H., JOHANSSON, S.: Lancet, 1967. 2 1105.  
'Diagnosis of Allergy by an *in vitro* test for Allergen antibodies'.

- WRAITH, D.G., MERRETT, J., ROTH, A., YMAN, L., MERRETT, T.G.:  
Clinical Allergy, 1979. 9 25. 'Recognition of food-allergic patients and their allergens by the RAST technique and clinical investigations'.
- WUTHRICH, B., BAUMANN, E., FRIES, R.A., SCHNYDER, U.W.:  
Clin. Allergy, 1981. 11 147. 'Total and specific IgE in atopic twins'.
- YOUNOSZAI, M.K., RANSHAW, J.: J.Nutrition, 1973. 2 454.  
'Gastrointestinal growth in the foetus and suckling rat pup'.
- ZAR, J.H. (Editor): Biostatistical Analysis, 1974. Prentice-Hall  
Biological Sciences Series.